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CHAPTER ONE

Role of Macrophage Polarization in Tumor Angiogenesis and Vessel Normalization: Implications for New Anticancer Therapies

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Contents

Abstract

Angiogenesis, the formation of new capillary blood vessels from preexisting vasculature, is one of the hallmarks of cancer that is pivotal for tumor growth and metastasis. Tumor vessels are known to be abnormal, with typically aberrant, leaky and disordered vessels. Thus, the combination of angiogenesis inhibition and vessel normalization is a potential strategy for anticancer therapy. The solid tumor is composed of not only cancer cells, but also the nonmalignant resident stromal cells, such as bone-marrow-derived cells (BMDCs) and cancer-associated fibroblasts (CAFs). Tumor-associated macrophages (TAMs) are the most abundant cell components of BMDCs, which play a significant role in promoting tumor progression. Accumulating evidences from both patient biopsies and experimental animal models have shown that TAMs function in tumor angiogenesis and vessel abnormalization in a density- and phenotype-dependent manner.

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This chapter will discuss the evidence for the factors and signaling pathways that are involved in macrophage recruitment and polarization in the tumor microenvironment, and it summarizes the role and underlying molecular mechanisms of macrophage polarization in tumor angiogenesis and vessel normalization. In addition, an overview of the potential of targeting TAM polarization for anticancer therapy will be provided.

1. INTRODUCTION

Angiogenesis, the formation of new capillary blood vessels from preexisting vasculature, is one of the hallmarks of cancer (Hanahan and Weinberg, 2000). The tumor cell population gained the most attention so far due to its capability of secreting proangiogenic factors that are critical in initiating tumor angiogenesis. However, tumor is not an island but rather an ensemble performance of nonmalignant resident stromal cells (also called tumor microenvironment), which includes cancer-associated fibroblasts (CAFs), endothelial cells, bone-marrow-derived cells (BMDCs), and extracellular matrix (ECM) (Ahn and Brown, 2008; Chan et al., 2009; Joyce, 2005). Among the tumor microenvironment, BMDCs are the major stromal cell population, which represents about 15–20% of total cells in solid tumors (Du et al., 2008). Several findings demonstrate that BMDCs are directly proportional to angiogenesis within the development of tumor (Balkwill and Coussens, 2004; Schmid et al., 2011; Yang et al., 2004, 2008), thus suggesting their important role in regulating tumor angiogenesis. BMDCs constitute extremely heterogeneous populations, which consist of CD45+ vascular modulatory cells, endothelial progenitor cells (EPCs), and pericyte progenitor cells (PPCs) (De et al., 2005; Du et al., 2008; Grunewald et al., 2006; Kopp et al., 2006; Lyden et al., 2001). CD45+ vascular modulatory cells make up the largest group of BMDCs. Such cells consist of several subtypes, including tumor-associated macrophages (TAMs) and immature monocytes including Tie2+ monocytes (TEMs), VEGFR1+ hemangiocytes, and CD11b+ myeloid cells (Du et al., 2008). In the tumor microenvironment, EPCs can incorporate into the vasculature and mature into endothelial cells, while PPCs can envelop blood vessels and differentiate into pericytes and vascular smooth muscle cells. Of the multiple stromal cell types in solid tumors, TAMs are most significant for fostering tumor angiogenesis and progression (Condeelis and Pollard, 2006). It was shown that the level of infiltrating macrophages is positively correlated with tumor angiogenesis and poor prognosis in cancer patients (Lewis and Pollard, 2006). Macrophage depletion in mouse tumor models results in decrease of vascular density. Conversely, overexpression of the colony-stimulating factor-1

(CSF-1) induces the enhancement of macrophage recruitment, causing an increase of tumor angiogenesis (Lewis and Pollard, 2006; Lin et al., 2001).

Angiogenesis is well known to promote tumor growth and metastasis. However, unlike the healthy vasculature, tumor vessels are highly chaotic, poorly organized and dysfunctional (vessel abnormalization), due to the excessive production of proangiogenic factors (De et al., 2011; Jain, 2005). These abnormalities of tumor vessels result in a hypoxic tumor microenvironment and represent physiological barriers for the delivery of cancer therapeutic agents (Fokas et al., 2012). The restoration of the balance between pro- and antiangiogenic factors production in tumor microenvironment may play a pivotal role in modulating the normalization of the structure of tumor blood vessels (Jain, 2005). Some important molecules, such as vascular endothelial growth factor (VEGF) (Jain, 2005), placental growth factor (PlGF) (Fischer et al., 2007; Fokas et al., 2012; Hedlund et al., 2009; Van et al., 2010), platelet-derived growth factors (PDGFs), angiopoietins, HIF-prolyl hydroxylases (PHD) (De et al., 2011), Rgs5 (Hamzah et al., 2008), CD160 receptor (Chabot et al., 2011), nitric oxide (NO) and EGF receptor (Kashiwagi et al., 2008), derived from either tumor cells or stromal cells, were reported to participate in regulating vessel normalization. In addition, intracellular signaling pathways, such as PI3K/mTOR pathway (Fokas et al., 2012; Qayum et al., 2012), are also involved in regulating vessel normalization. Understanding molecular mechanisms of vessel normalization may ultimately lead to more effective therapeutic strategies against cancer.

Interestingly, VEGF ablation in inflammatory cells promotes vessel normalization and vessel maturation (Stockmann et al., 2008), suggesting that inflammatory cells, such as macrophages, play a significant role in these processes. Depending on the activation states induced by the microenvironment, macrophages can be designated as either classically activated (M1 phenotype) or alternatively activated (M2 phenotype) (Sica et al., 2008). The different phenotypes of macrophages may exhibit opposing effects in blood vessels modeling and tumor progression. M2, rather than M1, macrophages were defined as the proangiogenic phenotype due to their ability to secrete factors that promote angiogenesis (Lamagna et al., 2006). Moreover, M2 macrophages induce vessel abnormalization, while M1 macrophages lead to vessel normalization (Rolny et al., 2011). These evidences suggest the significant role of TAM polarization from the M2 phenotype to the M1 phenotype in the regulation of tumor angiogenesis and vessel normalization. Thus, tumor vascular network, including angiogenesis and vessel normalization, is affected by the dynamic changes in macrophages phenotypes.

The current review will summarize a comprehensive overview about the basic biology of macrophages, monocytes recruitment and the polarization of macrophages in the tumor microenvironment, as well as the function and underlying mechanisms of TAM polarization in the regulation of tumor angiogenesis and vessel normalization. Furthermore, the potential of targeting TAMs in the tumor microenvironment for anticancer therapy by tumor angiogenesis inhibition and vessel normalization will also be discussed.

2. MACROPHAGES AND THEIR PHENOTYPES

Macrophages are a major population in the mononuclear phagocytic lineage deriving from bone marrow progenitor cells (Doulatov et al., 2010). In contrast to the other mononuclear phagocytic lineages, such as dendritic cells (DCs), macrophages have a longer lifespan (from hours to possibly years) and display proteolytic and catabolic activities, such as phagocytosis, which allow them to be highly effective in the ingestion of pathogens, in the clearance of dead cells and debris, and in the remodeling of tissues (Galli et al., 2011). Macrophages can be subcategorized according to their anatomical locations, and tissue-specific resident macrophages include osteoclasts in bone, alveolar macrophages in lung, histiocytes in interstitial connective tissues, microglia in brain, Kupffer cells in liver and splenic macrophages in spleen. However, they may have similar functional abilities following the appropriate stimuli (Galli et al., 2011). To distinguish them from other cell types, macrophages can be characterized by their specific markers. In mice, macrophages express CD11b, F4/80, and CSF-1R, but not Gr1. In human, they display specific expression of CD68, CD163, CD312, CD115 and CD16 (Qian and Pollard, 2010). On the other hand, macrophages themselves are also heterogeneous, and they can be divided into M1 and M2 phenotypes according to the Th1/Th2 dichotomy (Gordon and Taylor, 2005). M1 phenotype macrophages induce type I helper T cell (Th1) response. This population of macrophages is activated by bacterial moieties, such as lipopolysaccharide (LPS), and Th1 cytokine interferon-gamma (IFN-γ). In contrast, M2 phenotype macrophages are involved in type II helper T cell (Th2) response, and are activated by cytokines secreted from Th2 cell, such as interleukin 4 (IL-4) and IL-13. These two phenotypes of macrophages can be characterized by their different patterns of gene and protein expression (Table 1.1). For example, M1 macrophages display a high expression of major histocompatibility complex (MHC) class II, IL-12 and tumor necrosis factor-alpha (TNF-α), and also generate reactive

Table 1.1 Genes and molecules differentially expressed in M1 and M2 phenotype macrophages

Highly expressed genes and molecules in M1 macrophages	Membrane receptors	*IL2RA*, *IL15RA* (Martinez et al., 2006); TLR2, TLR4, CD16, CD32, CD64, CD80, CD86, IL-1R type I, CCR7 (Mantovani et al., 2002)
	Cytokines and growth factors	*IL15*, *ECGF1* (Martinez et al., 2006); TNF-α, IL-1, IL-6, IL-12 and IFN-γ (Mantovani et al., 2002)
	Chemokines	*CCL15*, *CCL19*, *CCL20* (Martinez et al., 2006); CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5 (Mantovani et al., 2002)
	Effector molecules	iNOS, ROI (Mantovani et al., 2002)
Highly expressed genes and molecules in M2 macrophages	Membrane receptors	*CD209*, *CD36*, *MS4A4A*, *MS4A6A TLR5*, *CLECSF13*, *TGFBR2*, *CXCR4* (Martinez et al., 2006); Scavenger receptor A/B, CD163, CD206, CD14, CD23, IL-1R type II, IL7R, CCR2, CXCR1 and CXCR2 (Mantovani et al., 2002)
	Cytokines and growth factors	*IGF1* (Martinez et al., 2006); IL-1ra, IL-10, IL-4 and IL-13 (Mantovani et al., 2002); MSF (Solinas et al., 2010)
	Chemokines	*CCL13*, *CCL23* (Martinez et al., 2006); CCL17, CCL22, CCL24, CCL16 and CCL18 (Mantovani et al., 2002)
	Effector molecules	Arginase I (Mantovani et al., 2002)

CLECSF13, C-type lectin superfamily member 13; *ECGF1*, endothelial cell growth factor 1; *IGF1*, insulin-like growth factor 1; *IL2RA*, interleukin 2 receptor α chain; *MS4A4A*, membrane-spanning 4-domains, subfamily A, member 4A; *TGFBR2*, transforming growth factor-beta (TGF-β) receptor type 2.
CCR7, chemokine (C–C motif) receptor; CXCL8, chemokine CXC motif ligand 8; CXCR1, C-X-C chemokine receptor 1; IL-1R type I, interleukin 1 receptor type I; ROI, reactive oxygen intermediates; TLR2, Toll-like receptor 2.

oxygen species (ROS) and NO (Qian and Pollard, 2010). Conversely, M2 macrophages show high expression of mannose receptor (CD206), galactose receptor, arginase I, IL-10, IL-1 decoy receptor, and IL-1RA, and low expression of IL-12 (Biswas and Mantovani, 2010; Gordon and Taylor, 2005; Mantovani et al., 2002; Mantovani, 2008). Recent study demonstrated that M2 macrophages specifically express and secrete migration-stimulating factor (MSF), a truncated isoform of fibronectin that is considered as a new marker for M2 macrophages (Solinas et al., 2010). Given their different gene expression profiles, the functions of M1 and M2 macrophages are also different. M1 macrophages are able to kill microorganisms and tumor cells by activating immune responses. By contrast, M2 macrophages are generally considered as immunosuppressive cells, which promote angiogenesis, and tissue remodeling and repair (Mantovani et al., 2002). TAMs are always biased toward the M2 phenotype (Mantovani and Sica, 2010). Monocytes recruitment into tumors, the polarization of macrophages in tumors and its functions and underlying mechanisms in promoting tumor malignancy and in remodeling vascular network including angiogenesis and vessel normalization, are discussed below.

3. RECRUITMENT OF MONOCYTES INTO TUMORS

It has been shown that there are higher numbers of macrophages in tumor tissues than surrounding normal tissues (Murdoch et al., 2008). These cells initially originate from monocytes that extravasate across the tumor vessels from blood and differentiate into TAMs following their recruitment into tumors. The chemoattractants including chemokines and cytokines secreted from both malignant and stromal cells that contribute to the recruitment of monocytes (Murdoch et al., 2008, 2004). Among these chemoattractants, chemokine (C–C motif) ligand 2 (CCL2, also known as monocyte chemoattractant protein-1, MCP-1) is the main one (Bottazzi et al., 1983, 1992; Loberg et al., 2007; Murdoch et al., 2008), which is widely expressed in multiple types of tumors (Bingle et al., 2002; Loberg et al., 2006; Murdoch et al., 2008). Both the experimental studies using CCL2 overexpression and knockdown approaches, and the investigation on human tumor biopsies support the concept that CCL2 plays a pivotal role in monocytes recruitment into tumors (Murdoch et al., 2004). Recent studies showed that CCL2 is responsible for the recruitment of inflammatory monocytes (which express CCR2, the receptor for CCL2), which in turn promotes tumor metastasis. Inhibition of CCL2–CCR2 signaling or

depletion of tumor cell-derived CCL2 blocks the recruitment of monocytes, inhibits tumor metastasis and prolongs the survival of tumor-bearing mice (Qian et al., 2011). Other chemokines, such as CCL3 (macrophage inflammatory protein-1α, MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CXCL8 (IL-8), and CXCL12 (stromal cell-derived factor 1, SDF-1) (Murdoch et al., 2004, 2008; Scotton et al., 2001), S100A8 and S100A9 (Hiratsuka et al., 2006, 2008), are also involved in monocytes recruitment into tumors or/and premetastastic sites. Furthermore, these chemokines directly stimulate monocytes to express some related molecules/chemoattractants that contribute to not only monocyte recruitment but also tumor progression. For example, CCL5 promotes the expression of CCL2, CCL3, CCL4, CXCL8 and CCR1 in human monocytes, thus induces a positive amplification loop for chemokines production in tumors that accelerate monotytes recruitment (Locati et al., 2002; Murdoch et al., 2004).

In addition to chemokines, cytokines are also implicated in the recruitment of monocytes into tumors. VEGF and macrophage colony stimulating factor (M-CSF) are two prominent cytokines that are commonly produced by tumors. They promote monocytes recruitment by acting tyrosine kinase receptors (Mantovani et al., 2004). It was shown that CSF-1 and its receptor, CSF-1R, are overexpressed in a wide variety of human tumors, and they are positively correlated with macrophage infiltration in tumors (Murdoch et al., 2004). Studies in mouse models suggested that overexpression of CSF-1 in tumors promotes TAM infiltration (Dorsch et al., 1993), while CSF-1 knockout decreases this infiltration (Lin et al., 2001). In addition to its proangiogenic effect, VEGF also functions as a chemotactic factor that is responsible for monocytes recruitment. It was shown that VEGF promotes monocytes migration through its receptors, VEGF-R1 (Barleon et al., 1996; Sawano et al., 2001) and VEGF-R2 (Dineen et al., 2008). VEGF-R1 depletion in macrophages induces the decrease of macrophage migration in response to VEGF stimulation (Hiratsuka et al., 1998). In vivo studies demonstrated that the elevated VEGF expression is positively correlated by the number of macrophage infiltration in breast cancer (Leek et al., 2000). Furthermore, recent studies suggested that VEGF expression in skin cancers is also correlated with macrophage infiltration (Linde et al., 2012). These findings highlight the important role of M-CSF and VEGF in monocytes recruitment into tumors.

More recent studies demonstrated that some other factors, such as tissue factor (TF), transcription factor and chitinase, are also involved in monocytes recruitment. TF expression in tumor is usually correlated with metastasis in

experimental settings and poor prognosis in human tumors (Gil-Bernabe et al., 2012; Laubli and Borsig, 2010; Palumbo and Degen, 2007). TF-induced clot formation indirectly enhances tumor cell survival and metastasis by recruiting macrophages. Genetic or pharmacologic inhibition of coagulation, by induction of TF pathway inhibitor expression, abrogates macrophage recruitment and tumor cell survival (Gil-Bernabe et al., 2012). These data suggested that TF is a significant player in macrophage recruitment into tumors. The expression of forkhead Box m1 (Foxm1) transcription factor is increased in many human tumors (Kalin et al., 2011; Myatt and Lam, 2007), and its expression is associated with poor prognosis in cancer patients (Xia et al., 2012a, 2012b). Balli et al. (2011) generated a mouse model with macrophage-specific Foxm1 deletion, and found that this deletion decreases macrophage recruitment into tumors by downregulating some chemokines, such as IL-6, CCL3 and MIP-2, and chemokine receptors CXCR1 and CXCR4, suggesting Foxm1 transcription factor is required for macrophage migration. Chitinase 3-like 1 (CHI3L1) is a member of chitinase family, which is expressed in several types of human cancers (Eurich et al., 2009; Johansen et al., 2006). CHI3L1 expression in tumors is positively associated with macrophage infiltration and poor prognosis. Overexpression of CHI3L1 in macrophages enhances their abilities of migration in vitro, while CHI3L1 antibody has the opposite effect. Mechanistic studies demonstrated that CHI3L1-induced macrophage recruitment is mediated by the upregulation of IL-8 and CCL2, and activation of mitogen-activated protein kinase (MAPK) signaling pathway (Kawada et al., 2011). These studies provide further insights into the mechanisms of in monocytes recruitment into tumors.

4. MACROPHAGES AND MACROPHAGE POLARIZATION IN THE TUMORIGENESIS

4.1. Macrophages and Cancer

Studies from patient biopsies strongly suggest that macrophages contribute to tumorigenesis. Table 1.2 lists the current knowledge on the correlation between TAM levels and patient survival in a wide range of human tumors. The clinical data suggest that TAM density is correlated with poor prognosis in most tumors. These clinical observations are well supported by experimental studies using macrophages depletion or overexpression approaches. For example, genetic ablation of the M-CSF in different murine tumor models, such as the polyoma middle T (PyMT) oncoprotein breast cancer model, the colon cancer spontaneous model (Oguma et al., 2008)

Table 1.2 The correlation between TAMs density and prognosis or angiogenesis in different forms of human cancer

Tumor type	Prognosis	Angiogenesis	References
Stomach	Good	No	(Ohno et al., 2003)
Colorectal	Good	No	(Funada et al., 2003)
Melanoma	No correlation	No	(Piras et al., 2005)
Ovary	Poor	Increase	(Orre and Rogers, 1999)
Cervix	Poor	Increase	(Salvesen and Akslen, 1999)
Lung (NSCLC)	Poor	Increase	(Koukourakis et al., 1998)
Glioma	Poor	Increase	(Nishie et al., 1999)
Breast	Poor	Increase	(Leek et al., 1999)
Prostate	Poor	Increase	(Lissbrant et al., 2000)
Endometrial	Poor	Increase	(Ohno et al., 2004)
Bladder	Poor	Increase	(Hanada et al., 2000)
Kidney	Poor	Increase	(Hamada et al., 2002)
Squamous cell carcinoma	Poor	Increase	(Koide et al., 2004)
Malignant uveal melanoma	Poor	Increase	(Makitie et al., 2001)
Follicular lymphoma	Poor	No	(Farinha et al., 2005)
Hepatocellular	Poor	Increase	(Zhu et al., 2008)
Thyroid	Poor	No	(Ryder et al., 2008)
Cholangiocarcinoma	Poor	No	(Subimerb et al., 2010)
Sarcoma	Poor	No	(Fujiwara et al., 2011)
Lymphoma	Poor	No	(Harris et al., 2012)
Pancreatic	Poor	Increase	(Kurahara et al., 2012)

"No" indicates without detection. NSCLC, non-small-cell lung carcinoma.

and the osteosarcoma xenotransplant model (Kubota et al., 2009), significantly reduces the macrophage density in these tumors, which in turn inhibits tumor growth and progression (Lin et al., 2001). In contrast, overexpression of CSF-1 in wild-type tumors increases macrophage recruitment and accelerates tumor growth and metastasis. Furthermore, studies using small interfering RNAs or antibodies to inhibit CSF-1 expression in MCF-7 xenografts confirmed these findings, showing that TAMs depletion is associated with the reduction of tumor growth and angiogenesis (Abraham et al., 2010; Aharinejad et al., 2004; Paulus et al., 2006). In addition, specific depletion of macrophages with clodronate-encapsulated liposomes also demonstrates a significant growth reduction of several forms of tumor (Qian and Pollard, 2010). It should be noted, however, that although the experimental and clinical data largely support the hypothesis that macrophages promote tumor growth and progression, there are exceptions. As shown in Table 1.2, a few tumors, such as stomach and colorectal tumor, have the correlation of high TAM density with good prognosis. In animal models, similar observations were found for liver macrophages, which have the function to kill the circulating tumor cells, and depletion of such cells induces the enhancement of metastasis (van der Bij et al., 2005b) and tumor differentiation (Oosterling et al., 2005). Taken together, these data suggest that TAMs may also exhibit antitumorigenic properties (Bingle et al., 2002; van der Bij et al., 2005a) although they are primarily considered as protumorigenic (Condeelis and Pollard, 2006; Pollard, 2004). The different functions of TAMs may depend on their activation states induced by the different tumor microenvironments (Watkins et al., 2007). Following their recruitment into the tumor site, monocytes can be "educated" by the tumor microenvironment and differentiate into different phenotypes, including M1 and M2 macrophages (Sica et al., 2008). Such differences of the TAM phenotype in different tumors may explain their distinct functions in modulating tumorigenesis and progression.

4.2. Macrophage Polarization in the Tumor Microenvironment

In regressing, nonprogressing or early stage of tumors, TAMs mainly resemble the M1 phenotype, while in malignant and advanced tumors, TAMs are biased toward the M2 phenotype (Biswas and Mantovani, 2010; Chen et al., 2011; Lamagna et al., 2006; Qian and Pollard, 2010; Rolny et al., 2011). These findings further suggest that the phenotype of TAMs can be modified by microenvironmental triggers in the tumor. A recent study showed that TAM phenotype is switched from M1 to M2 during the tumor growth

(Chen et al., 2011), which not only supports the concept that macrophage phenotypes can be changed according to different tumor microenvironments but also provides a new insight into the dynamic nature of TAM phenotypes during the tumor growth. Given the opposing effects of M1 and M2 macrophages for tumor progression, there is a great deal of interest in elucidating the factors that regulate macrophage polarization in the tumor microenvironment, as well as their underlying molecular mechanisms.

4.2.1. Macrophage Polarization-Related Factors in the Tumor Microenvironment

Macrophage polarization-related factors have been recently reviewed, and these include M2 phenotype polarization factors, such as IL-4, IL-10, IL-13, IL-21, IL-33, CCL2 and transforming growth factor-β (TGF-β), and M1 phenotype polarization factors, such as IFN-γ and LPS (Biswas and Mantovani, 2010; Galli et al., 2011). Macrophage polarization in the tumor microenvironment is regulated by a number of factors including growth factors, chemokines, interleukins and other molecules (Table 1.3). We herein put an emphasis on summarizing recent research progress in this topic.

VEGF plays an important role in tumor angiogenesis and progression. A recent study demonstrated that VEGF-A-induced skin carcinogenesis depends on the alternative activation of macrophages, suggesting a role for VEGF-A in M2 macrophage polarization. However, this effect is indirect since VEGF-A itself does not induce M2 macrophage polarization in vitro (Linde et al., 2012). Similar to VEGF, adrenomedullin (ADM) is another angiogenic peptide, and it is widely expressed in a variety of tumors (Zudaire et al., 2003). In melanoma, TAMs are the major source of ADM, and TAM-derived ADM upregulates CD206 expression and arginase I production, and downregulates inducible NO synthase (iNOS) production, thus shewing macrophages toward the M2 phenotype in an autocrine manner (Chen et al., 2011).

As mentioned above, CCL2 is the main chemoattractant that is responsible for the recruitment monocytes into the tumor sites (Murdoch et al., 2008). IL-6, one of the most abundant cytokines in the tumor microenvironment, is typically expressed by cancer cells (Siegall et al., 1990). Interestingly, expression of CCL2 and IL-6 in myeloid cells is induced in a reciprocal manner. CCL2 expression in macrophages shows a twofold increase upon IL-6 treatment, and IL-6 expression exhibits fivefold upregulation upon CCL2 stimulation. Therefore, CCL2 and IL-6 induce an amplification loop that affects tumor microenvironment, including TAMs and cancer cells.

Table 1.3 The factors involved in regulating TAM polarization

TAM polarization	Factor types	Major factors and References
M2 polarization	Growth factors	VEGF (De, 2012; Linde et al., 2012); ADM (Chen et al., 2011); PlGF (Rolny et al., 2011); TGF-β, GM-CSF (Biswas and Mantovani, 2010; Galli et al., 2011)
	Chemokines	CCL2 (Roca et al., 2009)
	Interleukins	IL-4, IL-10, IL-13, IL-21, IL-33 (Biswas and Mantovani, 2010; Galli et al., 2011); IL-6 (Roca et al., 2009)
	Others	DcR3 (Tai et al., 2012); ANG-2 (Coffelt et al., 2010)
M1 polarization	HRG (Rolny et al., 2011); AMA (Chen et al., 2011); Celecoxib (COX-2 inhibitor) (Nakanishi et al., 2011); MENK (Chen et al., 2012a)	

GM-CSF, granulocyte/macrophage colony stimulating factor.

Moreover, each member of this loop can induce macrophage polarization to M2 phenotype by upregulation of CD206+ and CD14+/CD206+ cell populations, via the inhibition of caspase-8 cleavage and enhanced autophagy (Roca et al., 2009).

Decoy receptor 3 (DcR3) is a soluble protein belonging to the TNF receptor family and that is overexpressed in cancer cells. DcR3 expression in tumors is correlated with poor prognosis in a variety of tumors. DcR3 modulates TAMs polarization to M2 phenotype by downregulating MHC class II expression through an epigenetic mechanism (Tai et al., 2012). Angiopoietin 2 (ANG-2) is an angiogenic peptide released by endothelial cells, which plays an essential role in modulating angiogenesis and vascular homeostasis (Augustin et al., 2009). Besides its angiogenic function, ANG-2 increases the expression of M2-like genes, such as IL-10, CD206 and CCL17, in macrophages in vitro. Furthermore, in vivo studies showed that ANG-2 overexpression in a mouse tumor model induces higher vascular density and M2 TAM infiltration (Coffelt et al., 2010). Altogether, these findings shed light on the critical factors skewing macrophages toward M2 phenotype in the tumor microenvironment, which in turn promote angiogenesis, tumor growth and metastasis, and also provide some potential targets for anticancer therapies.

The potential molecules involved in TAMs polarization from M2 to M1 phenotype are also documented. Histidine-rich glycoprotein (HRG) is a heparin-binding protein, and its expression is downregulated in tumors (Rolny

et al., 2011). Both pharmacological and genetic approaches indicate that HRG inhibits tumor angiogenesis, growth and metastasis by promoting immune responses (Rolny et al., 2011; Tugues et al., 2012), especially by skewing TAM polarization away from the M2 phenotype to a tumor-inhibiting M1 phenotype via downregulation of PlGF (Rolny et al., 2011). Adrenomedullin22-52 (AMA), an antagonist of ADM receptors, inhibits tumor growth by inducing M2 macrophages polarization to M1 phenotype through downregulation of ADM expression in an autocrine manner (Chen et al., 2011). Cyclooxygenase-2 (COX-2) is constitutively expressed in various tumors, and TAMs are considered as the major source of COX-2 (Inaba et al., 2003). It has been shown that celecoxib, an inhibitor of COX-2, induces TAM polarization from M2 to M1 phenotype in intestinal tumors (Nakanishi et al., 2011). Metenkephalin (MENK) is an endogenous neuropeptide, which has the ability to inhibit tumor growth and metastasis (Kuniyasu et al., 2010). The expression of MENK in tumors, such as colorectal cancer, is associated with tumor-infiltrating immune cells (Ohmori et al., 2009). It was recently demonstrated that MENK induces TAM polarization to M1 phenotype, as indicated by the downregulation of CD206 and arginase I (Chen et al., 2012a). Taken together, these evidences provide novel insights into potential targets for efficient tumor therapeutic strategies through TAM polarization from M2 to M1 phenotype.

4.2.2. *Molecular Mechanisms of Macrophage Polarization in the Tumor Microenvironment*

Nuclear factor-kappaB (NF-κB) signaling is a key regulator that links inflammation and cancer (Ben-Neriah and Karin, 2011; Grivennikov et al., 2010). The potential role of NF-κB signaling in macrophages polarization was validated in both in vitro and in vivo experiments. It was shown that macrophages polarization to M2 phenotype requires NF-κB activation. When NF-κB signaling is specifically inhibited by blocking IκB kinase (IKK)β, the major activator of NF-κB, TAMs are switched to M1 phenotype, which in turn inhibits tumor growth (Hagemann et al., 2008). On the other hand, overexpression of p50 NF-κB overexpression in TAMs inhibits M1 inflammatory responses and antitumor resistance (Saccani et al., 2006). It was recently demonstrated that Wnt5a induces a negative feedback loop of NF-κB activation in macrophages and has the ability to skew macrophage polarization to M2 phenotype in breast cancer patients (Bergenfelz et al., 2012). Notch signaling is another important mediator in the determination of M1 versus M2 polarization of TAMs. Notch signaling activation induces macrophage polarization to M1 phenotype, whereas blockade

of this signaling causes macrophage polarization to M2 phenotype (Wang et al., 2010). These studies support the important function for NF-κB and Notch signaling pathways in macrophage polarization. Furthermore, several transcription factors, such as signal transducer and activator of transcription 6 (Stat6), peroxisome proliferator-activated receptor-gamma (PPARγ), and c-Myc, also play important roles in this process (Bouhlel et al., 2007). Stat6 is a facilitator of PPARγ-regulated gene expression in macrophages (Szanto et al., 2010), which is responsible for epigenetic changes mediated by the histone demethylase Jumonji domain containing 3 (Jmjd3), which in turn leads to M2 polarization (Ishii et al., 2009). c-Myc is required for the polarization of macrophages into M2 phenotype by inducing a significant set of M2-related genes through Stat6 and PPARγ pathways (Pello et al., 2012).

Autophagy is a key cellular process widely occurring in eukaryotic cells (Reggiori and Klionsky, 2002). In macrophages, autophagy is an important regulator of innate immunity (Xu et al., 2007). Moreover, the induction of autophagy is pivotal for the survival and differentiation of monocytes (Zhang et al., 2012b). CCL2 and IL-6 treatment induces macrophages skewing to M2 phenotype, a process that is also accompanied by induction of autophagy. Inhibition of caspase-8 can mimic similar functions in macrophages. However, combination treatment with autophagy inhibitors or lysosomal protease inhibitors, and caspase-8 inhibitor results in a significant decrease in CD206+ and CD14+/CD206+ cell populations when compared with caspase-8 inhibitor treatment alone, in agreement with the concept that autophagy plays a significant role in M2 macrophage polarization (Roca et al., 2009). The mTOR kinase is a major regulator of autophagy (Jung et al., 2010), and mTOR positively regulates immune cell activation (Weichhart and Saemann, 2008). The TSC2–mTOR signaling pathway is critical for macrophage polarization, as demonstrated by the fact that when this pathway is inhibited by rapamycin or activated by TSC2 knockdown, LPS-stimulated monocytes are induced to differentiate into M1 or M2 macrophages, respectively (Chen et al., 2012b).

These findings highlight that the characterization of the molecular mechanisms involved in macrophage polarization within the tumor microenvironment is essential for improving our understanding of cancer biology. In summary, upon stimulation by different factors in the tumor microenvironment, macrophage gene transcription is regulated through specific signaling pathways and transcription factors, thus leading to macrophage polarization to M1 or M2 phenotypes to exert different functions in tumor angiogenesis, growth and progression (Fig. 1.1).

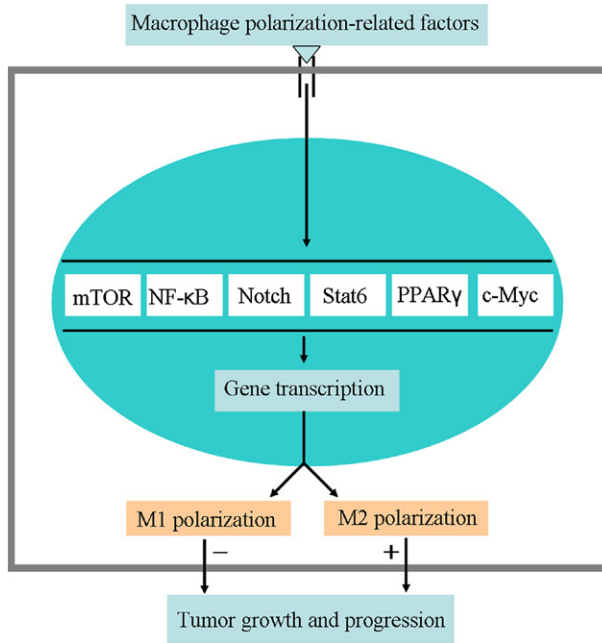

Figure 1.1 ***Schematic representation of signaling pathways in macrophage polarization.*** Macrophage polarization-related factors in the tumor microenvironment exert their effects through macrophage surface receptors to trigger intracellular signaling pathways, such as mTOR, NF-κB, Notch, Stat6, PPARγ and c-Myc, which in turn induce gene transcription. By sensing the differently transcribed gene products, macrophages are polarized to classically activated (M1) or alternatively activated (M2) phenotype, which, respectively, inhibits and promotes tumor angiogenesis and progression. For color version of this figure, the reader is referred to the online version of this book.

5. ROLE OF TAMs AND THEIR POLARIZATION IN TUMOR ANGIOGENESIS

5.1. Role and Mechanisms of TAMs

In most tumors, blood vessels are dramatically increased during the transition from benign to malignant states, a process regarded as an angiogenic switch and that is influenced by the tumor microenvironment (Qian and Pollard, 2010). The potential role of macrophages in modulating tumor angiogenesis was first proposed by Sunderkotter et al. in 1991 (Sunderkotter et al., 1991). After that, a variety of studies have shown that TAMs are often found in the surrounding of blood vessels of solid tumors (Leek et al., 1996; Negus et al., 1997; Ohno et al., 2004). As shown in Table 1.2, studies in human tumors demonstrate a positive correlation between blood vessel density and the

number of TAMs in vessel areas (Leek and Harris, 2002; Onita et al., 2002). The proangiogenic function of TAMs was also thoroughly investigated in animal cancer models. Accumulating evidences show that TAM depletion results in the decrease of tumor angiogenesis (Lin et al., 2001, 2006), while TAM enhancement exhibits the opposing effect (Zhang et al., 2010). For example, it has been shown that genetic depletion of macrophages in PyMT mammary tumor model delays the angiogenic switch, whereas restoration of macrophage infiltration rescues the vessel phenotype (Lin et al., 2006).

In addition to the functional studies mentioned above, much interest has been given to the mechanistic insights on the proangiogenic function of TAMs. Hypoxia is a common feature of solid tumors, and macrophages are often attracted to the hypoxic areas of tumor site due to the secretion of hypoxia-induced chemoattractants by tumor cells. Such chemoattractants include VEGF, endothelin, endothelial monocyte activating polypeptide II (EMAP II) (Murdoch et al., 2004) and CCL2 (Murdoch et al., 2008). Once TAMs are attracted to the hypoxic areas, this microenvironment promotes the metabolic adaptation of TAMs to hypoxia by upregulating hypoxia-inducible factors (HIF)-1, HIF-2 and VEGF (Burke et al., 2002; Lewis and Pollard, 2006; Murdoch and Lewis, 2005). VEGF-A functions as a potent mitogen for endothelial cells by binding to VEGFR1 and VEGFR2 (Squadrito and De, 2011). Genetic studies showed that TAM-derived VEGF-A is essential for angiogenesis in the PyMT mammary tumors (Lin et al., 2007). Restoring VEGF-A expression in macrophage-deficient PyMT tumor model induces the increase of tumor angiogenesis (Lin et al., 2007). These data indicate that VEGF is a key regulator of the proangiogenic activity of TAMs. Further studies suggested that hypoxia also upregulates the expression and secretion of ADM by macrophages (Cejudo-Martin et al., 2002), which are often regulated by HIF and VEGF (Fang et al., 2009; Oladipupo et al., 2011). A recent study showed that TAM-induced endothelial cell migration and tubule formation are inhibited by treatment with an ADM neutralizing antibody (Chen et al., 2011). These findings demonstrate that ADM can function as a novel pivotal factor of TAMs in facilitating tumor angiogenesis. TAMs also have the ability to release a number of other proangiogenic factors, including growth factors (such as PlGF, basic fibroblast growth factor (bFGF), M-CSF, PDGF, heparin-binding epidermal growth factor (HB-EGF), macrophage-inhibitory factor (MIF), platelet activating factor (PAF) and TGF-β), and cytokines (such as IL-1, IL-8, TNF-α and MCP-1) (Dirkx et al., 2006; Fischer et al., 2007). Recent studies have increased our understanding about TAM-derived

factors involved in angiogenesis. In solid tumors, the hypoxic condition often induces apoptosis of tumor cells (Weinmann et al., 2004). The apoptotic tumor cells can upregulate prostaglandin E2 (PGE2) production from macrophages to promote angiogenesis (Brecht et al., 2011). Semaphoring 4D (Sema4D) is a proangiogenic molecule that acts through its receptor, plexin B1 (Conrotto et al., 2005). In the tumor microenvironment, TAMs are the major source of Sema4D, which is critical for tumor angiogenesis and vessel maturation, as demonstrated by the impaired angiogenesis and vessel maturation in Sema4D knockout mice (Sierra et al., 2008). In addition to producing proangiogenic factors in the hypoxic condition, TAMs also promote angiogenesis by downregulating the expression of angiogenesis inhibitors, such as vasohibin-2 (Shen et al., 2012).

Apart from the secretion of proangiogenic factors, TAMs also express a number of angiogenesis-modulating enzymes, such as COX-2, iNOS, and matrix metalloproteinases MMP-2, MMP-7, MMP-9, and MMP12 (Klimp et al., 2001; Lewis and Pollard, 2006). TAM-derived MMP-9 was shown to be crucial for angiogenesis development in estrogen-treated K14-HPV16 transgenic mice, a model of human cervical carcinogenesis (Giraudo et al., 2004). Cathepsin proteases were also implicated in human tumor progression (Mohamed and Sloane, 2006). In the tumor microenvironment, TAMs are the primary source of the high levels of cathepsin protease activity in pancreatic cancer and mammary tumor. Removal of TAM-derived cathepsin B or S in these tumors impairs tumor angiogenesis, suggesting their critical roles in mediating TAMs effects on angiogenesis (Gocheva et al., 2010).

The ability of TAMs to secrete angiogenic factors is regulated by specific signaling pathways or transcription factors. Flt-1 is a tyrosine kinase receptor that binds to VEGF-A/B and PlGF and promotes angiogenesis (Olofsson et al., 1998; Sawano et al., 1996). Flt-1 is expressed by both endothelial cells and TAMs, and Flt-1 deficiency in TAMs impairs tumor angiogenesis, thus suggesting an important role of Flt-1 in mediating TAM-induced angiogenesis (Kerber et al., 2008). Activation of the Stat3 transcription factor mediates the function of TAMs in angiogenesis by upregulating several proangiogenic factors, such as VEGF and bFGF (Kujawski et al., 2008). In macrophage and tumor cell coculture systems, the expression of proangiogenic factors VEGF-A and VEGF-C in macrophages is dramatically upregulated. This effect is significantly reduced when the NF-κB signaling pathway is inhibited (Wu et al., 2012). It was recently shown that TSC2–mTOR signaling pathway also plays an important regulatory function in this process. Tubule formation is reduced or increased when endothelial

cells are cocultured with macrophages treated with either rapamycin or TSC2 siRNA, respectively (Chen et al., 2012b).

In summary, when TAMs are attracted to the hypoxic areas of tumor site, they produce a large body of proangiogenic factors in addition to angiogenesis-modulating enzymes, under the regulation of specific signaling pathways (i.e. NF-κB and mTOR) and transcription factors (i.e. Stat3), which together or partly contribute to tumor angiogenesis (Fig. 1.2). On the other hand, TAMs may also promote tumor angiogenesis by affecting other components, such as cancer cells, in the tumor microenvironment. For example, the interaction of mouse breast cancer cells and TAMs leads to the upregulation of Fra-1, a member of the FOS transcription factor family, which in turn induces activation of the IL-6/JAK/Stat3 signaling pathway in TAMs. This leads to increased release of the proangiogenic factors MMP9, VEGF and TGF-β by cancer cells, thus promoting tumor angiogenesis (Luo et al., 2010).

5.2. Role of Macrophage Polarization

Although most studies reported that TAMs usually favor tumor angiogenesis, this effect is largely dependent on TAM phenotypes. Increasing evidence demonstrate that TAMs normally exhibit the M2 (proangiogenic) phenotype, which promotes endothelial cell proliferation and tumor angiogenesis (Lewis and Pollard, 2006). However, in some cases, such as in the

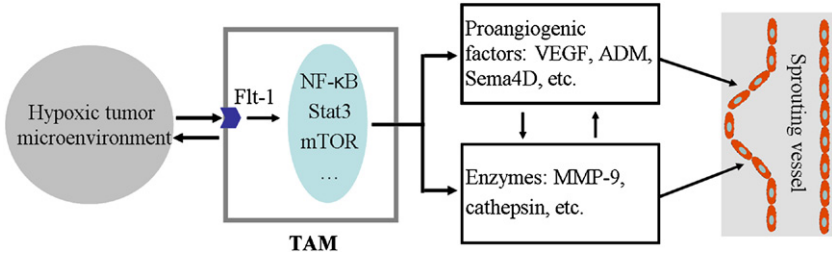

Figure 1.2 ***Proangiogenic function of macrophages and its molecular nature in tumors.*** Following the attraction into hypoxic areas in tumors, signals from tumor microenvironment induce macrophages activation by their cell surface receptors. During the process of macrophage activation, specific signaling pathways (including NF-κB and mTOR) and transcription factors (such as Stat3) are activated, leading to the secretion of proangiogenic factors (such as VEGF, PlGF, Sema4D, bFGF, IL-1, ADM and IL-8), and the production of angiogenesis-modulated enzymes (such as cathepsin proteases, COX-2 and matrix metalloproteinases MMP-2, MMP-7, MMP-9, and MMP-12). These proangiogenic factors and enzymes contribute to tumor angiogenesis. For color version of this figure, the reader is referred to the online version of this book.

early stage of tumors as well as in regressing and nonprogressing tumors, TAMs mainly resemble the M1 (antiangiogenic) phenotype, which causes angiogenesis inhibition and antitumor immune response, due to secretion of IFN-γ, IL-12 (Lamagna et al., 2006; Tsung et al., 2002) and/or TNF-α (Blankenstein et al., 1991; Lamagna et al., 2006). In vitro studies showed that the conditioned medium from resting macrophages inhibits endothelial cells proliferation, while the conditioned medium from activated macrophages displays the opposite effect (Pakala et al., 2002). Resting macrophages inhibit the proliferation of endothelial cells by releasing a variety of endothelial cell growth inhibitors, such as oncostatin M (OSM) and leukemia inhibitory factor (LIF) (Takashima and Klagsbrun, 1996). Furthermore, recent findings showed that TAM polarization from the M2 to the M1 phenotype by HRG or AMA inhibits tumor angiogenesis in vivo (Chen et al., 2011; Rolny et al., 2011). These evidences support the concept that the different macrophage activation states contribute to their opposite effects in regulating tumor angiogenesis. It is conceivable to target TAMs in order to limit tumor angiogenesis by modifying their activation status toward the antiangiogenic phenotype. Thus, the combination of the inhibition of TAM recruitment and their polarization from the M2 to the M1 phenotype should be considered as a new potential strategy in antiangiogenic studies and therapies.

6. ROLE OF TAMs AND THEIR POLARIZATION IN VESSEL NORMALIZATION

In the hypoxic microenvironment, TAMs produce an excess of angiogenic molecules, which not only promote tumor angiogenesis but also induce tumor vessel abnormalization. By impairing oxygen delivery, abnormal vessels trigger a vicious cycle of nonproductive angiogenesis, which creates a hostile microenvironment from where cancer cells escape through leaky vessels, thus rendering tumors less responsive to chemoradiation (De et al., 2011; Jain, 2005). Indeed, antiangiogenic vessel normalization strategies not only improve the chemotherapy drug delivery but also convert the malignant invasive and metastatic tumors into more benign and less aggressive cancers (De et al., 2011; Rolny et al., 2011). Thus, these strategies are gaining more attention, and are emerging as a new concept in cancer therapies (Jain, 2001).

Past work on treatment strategies aimed at vessel normalization mostly focused on the abnormalities of vascular endothelial cells and pericytes. However, the other components in the tumor microenvironment, such as

inflammatory cells (Stockmann et al., 2008), also play an important role in this process. It was recently reported that CCR2-dependent myeloid cells infiltration in tumors induces low response to chemotherapeutic agents, while CCR2 depletion reverses this phenomenon (Nakasone et al., 2012). Moreover, macrophages also have the ability to increase tumor resistance to chemotherapy, which is demonstrated by the fact that the survival of tumor-bearing mice is enhanced by the blockade of macrophage recruitment, in combination with chemotherapeutic agents (Denardo et al., 2011). Macrophage-induced chemotherapy resistance is, at least in part, mediated by the formation of abnormal and hypoperfused blood vessels, which limit the delivery of chemotherapeutic agents. Thus, it is conceivable that TAM depletion increases the efficacy of chemotherapy, which is due to the normalization of blood vessels, which enhances the blood flow to tumor and then increases the delivery of chemotherapeutic agents (De and Lewis, 2011). These findings reveal that myeloid cells, especially macrophages, function as an important player in blood vessel abnormalization, and limit the delivery of chemotherapeutic agents into tumors.

Recent studies have demonstrated that M2 macrophages depletion by clodronate treatment normalizes tumor blood vessels, while M1 macrophage depletion is not involved in this process, thus revealing an important role of TAMs and their polarization away from M2 to M1 phenotype in tumor vessels normalization (Huang et al., 2011; Rolny et al., 2011). This is the first indication that links TAM polarization to vessel normalization, thus implying that "re-education" of TAMs is a promising cancer therapeutic strategy by inducing tumor vessel normalization. The underlying molecular mechanisms of TAMs and their polarization in vessel normalization are discussed below and are summarized in Fig. 1.3.

PlGF, a VEGF family member, is not necessary for physiological angiogenesis but selectively promotes angiogenesis in diseased states (Carmeliet et al., 2001; Fischer et al., 2008). PlGF is highly expressed in tumors and exerts pleiotropic functions in promoting tumor angiogenesis and growth (Loges et al., 2009). Genetic or pharmacological blockage of PlGF induces vessel normalization in mouse spontaneous tumor models, without affecting the tumor blood vessel density (Van et al., 2010). Mechanistic studies in mice suggest that PlGF effects in tumor vessel disorganization are, in part, mediated by changes of TAM polarization. Macrophage loss of PlGF induces vessel maturation and reduces vessel leakage and remodeling, which in turn increase tumor oxygenation and response to chemotherapy (Rolny et al., 2011). PlGF blockage normalizes tumor vessels by shifting

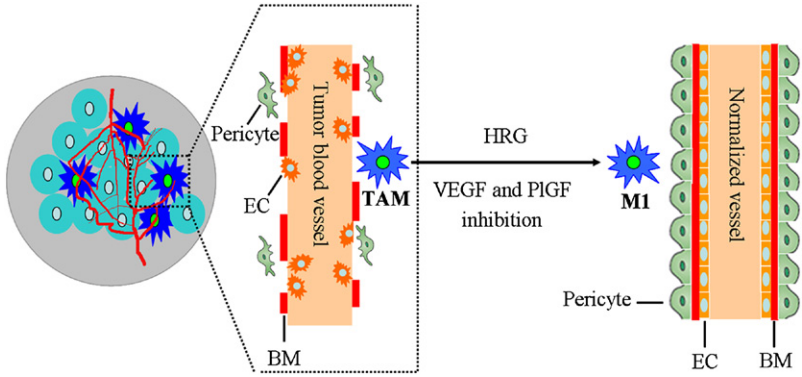

Figure 1.3 ***Role and underlying mechanisms of TAMs and their polarization away from M2 to M1 phenotype in vessel normalization***. In established solid tumors, blood vessels always exhibit structural and functional abnormalities, which may be induced by the excess of proangiogenic factors derived from TAMs skewing to the M2 phenotype. Tumor vessel normalization is emerging as a new therapeutic strategy for cancer. Myeloid cells, such as macrophages, are involved in tumor vessel disorganization. Genetic depletion of VEGF in myeloid cells or PlGF in macrophages induces tumor vessel normalization. Furthermore, HRG as well as the blockade of PlGF or VEGF signals in TAMs contribute to TAM skewing toward the M1 phenotype and tumor vessel normalization. BM, basement membrane; EC, endothelial cell. For color version of this figure, the reader is referred to the online version of this book.

TAM phenotype from M2 to M1, without affecting TAM density (Rolny et al., 2011).

VEGF is expressed in TAMs (usually M2 phenotype) of both human and mouse mammary tumors (Qian and Pollard, 2010). Given the importance of VEGF for angiogenesis in health and disease, it has been become the prime target for antiangiogenic therapies in cancer (Ellis and Hicklin, 2008). VEGF inhibition not only inhibits tumor angiogenesis and tumor progression but also induces vessel normalization (Jain, 2005). The blockade of VEGF expression results in increased pericyte coverage and vessel maturation, and decreased tumor vessel permeability (Abramovitch et al., 1999; Tong et al., 2004; Weisshardt et al., 2012). Interestingly, myeloid cell-derived VEGF is also very important in this process, as demonstrated by the fact that VEGF deletion in these inflammatory cells promotes vessel normalization and maturation (Stockmann et al., 2008). Recent studies demonstrated that VEGF-A is a potent chemoattractant for macrophages and that the majority of macrophages recruited to VEGF-A-expressing tumors exhibit M2 phenotype, thus implying an important role of VEGF-A in macrophage recruitment and polarization (De, 2012; Linde et al., 2012). These data strongly support

a mechanism where TAMs regulates vessel normalization by the distinct expression and production of VEGF in M1 or M2 macrophages. Further studies suggest that VEGF inhibition induces tumor vessel normalization in part by the remodeling of the vascular basement membrane and the surrounding ECM (Vosseler et al., 2005; Winkler et al., 2004). It is well established that ECM influences tumor growth and progression and regulates vascular morphogenesis in tumors (Bauer et al., 2009). Integrins is a family of heterodimeric receptors that link the surface of cells to different ECM components, thus mediating the transduction of cell-ECM signals. Some integrins, such as α5β3, α5β5, α5β1 and α6β5, are upregulated in tumors (Joyce, 2005). β3-intergrin is a promoter of endothelial cell survival during tumor angiogenesis (Brooks et al., 1994), and it is expressed in macrophages (Friggeri et al., 2010; Zhang et al., 2012a). Additionally, β3-intergrin seems to play a significant role in macrophage polarization, as demonstrated by findings that β3-intergrin knockout mice display impaired muscle regeneration and increased fibrosis due to the infiltration of macrophages that are polarized to M2 phenotype (Zhang et al., 2012a). Furthermore, β3-intergrin-binding peptide has the ability to induce vessel normalization in spinal cord injury mouse mode (Han et al., 2010). However, more studies are needed to clarify if TAMs produce β3-intergrin, and the significance of TAM-derived β3-intergrin for macrophage polarization and vessel normalization in tumors.

7. CONCLUDING REMARKS

Given the significant role of angiogenesis in solid tumor growth and metastasis, antiangiogenic strategies offered a promising perspective for cancer therapies. Bevacizumab, a monoclonal antibody targeting VEGF, is the first antiangiogenic agent used in clinic. When administered as a single agent in patients with solid tumors, bevacizumab produces modest responses (Cobleigh et al., 2003; Yang et al., 2003), but this treatment does not have the benefits of long-term survival (Mayer, 2004). Interestingly, when administered in combination with chemotherapeutic agents, bevacizumab can produce an unprecedented increase in cancer patients survival compared with chemotherapy alone (Hurwitz et al., 2004). These findings highlight the relevance of anti-VEGF activity in tumor angiogenesis inhibition and vessel normalization, and also pave the way for the development of new strategies in antiangiogenic therapies. Tumor progression is modulated by the synergistic effects coming from both tumor cells and stromal cells. Tumor angiogenesis regulation by TAMs, the most abundant stromal

cells, has largely been investigated and was shown that macrophages depletion decreases tumor angiogenesis and progression (Lin et al., 2001, 2006), thus pointing at TAMs as a potential target for cancer therapies. Moreover, increasing evidence suggest that macrophage polarization away from the M2 phenotype to the M1 phenotype can be regarded as another important strategy for cancer therapies (Sica et al., 2008). Recent studies indicate that HRG functions in vivo to repress M2 macrophage skewing, which results in both tumor angiogenesis inhibition and vessel normalization, while PlGF displays the opposing effects (Rolny et al., 2011). A number of findings support the concept that TAMs are educated by tumor cells and tumor microenvironment, and "re-education" of TAMs is now emerging as a novel strategy for cancer therapies via tumor angiogenesis inhibition and vessel normalization (Fig. 1.4). However, much work remains

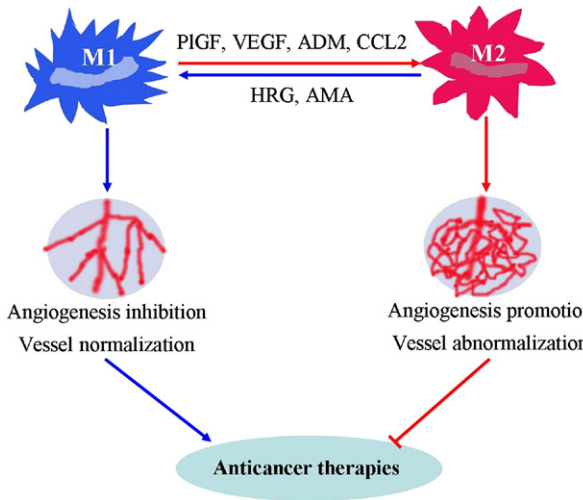

Figure 1.4 ***Cancer therapeutic strategy by targeting TAM polarization to inhibit tumor angiogenesis and normalize tumor vessels.*** TAMs exhibit the proangiogenic M2 phenotype, which not only promotes tumor angiogenesis but also leads to the abnormal tumor vessels. Conversely, M1 macrophages are considered as antiangiogenic and have the opposing effect in the modulation of vascular network. The phenotype of macrophages can be shifted based on the tumor microenvironment. For example, HRG and AMA, an antagonist of ADM receptors, or MENK can polarize TAMs away from the M2 to the M1 phenotype, which in turn inhibits tumor angiogenesis and/or normalizes tumor vessels. By contrast, proangiogenic factors such as PlGF, VEGF, ADM and CCL2 have the opposing effects. New cancer therapeutic strategies are being developed not only for inhibiting tumor angiogenesis but also for normalizing tumor vessels by the "re-education" of TAM polarization from an M2 phenotype to an M1 phenotype. For color version of this figure, the reader is referred to the online version of this book.

to be done in order to get a clear understanding of the communication between TAMs and tumor cells or other types of stromal cells, and how such communication processes contribute to TAM "re-education." In the meanwhile, more molecules that induce angiogenesis inhibition and vessel normalization by skewing TAMs toward M1 phenotype, as well as the mechanistic basis of these processes, need to be identified. Identifying the key elements in these anticancer therapeutic strategies will be the main challenge in the future.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare that they have no financial interests.

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CHAPTER TWO

New Insights into Desiccation-Associated Gene Regulation by *Lilium longiflorum* ASR during Pollen Maturation and in Transgenic *Arabidopsis*

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Contents

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Abstract

LLA23, a member of the abscisic acid-, stress-, and ripening-induced (ASR) protein family, was previously isolated from lily (*Lilium longiflorum*) pollen. The lily ASR is induced through desiccation-associated ABA signaling transduction in the pollen. ASRs are highly hydrophilic and intrinsically unstructured proteins with molecular masses generally less than 18 kDa. LLA23 is abundant in the cytoplasm and nuclei of both vegetative and generative cells of pollen grains. The protein in the nucleus and in the cytoplasm is partly regulated by dehydration. A dual role is proposed for LLA23, as a regulator and a protective molecule, upon exposure to water deficits. This chapter reviews the current state of literature on *Asr* genes, protein structure, function, and their responses to various stresses. In a study, a genome-wide microarray was used to monitor the expression of LLA23-regulated genes, focusing on the relationship between ASR-, glucose-, and drought-inducible genes, and outlined the difference and cross talk of gene expression among these signaling networks. A strong association was observed in the expression of stress-responsive genes and found 25 genes that respond to all three treatments. Highly inducible genes were also found in each specific stress treatment. Promoter sequence analysis of LLA23-inducible genes enabled us not only to identify possible known *cis*-acting elements in the promoter regions but also to expect the existence of novel *cis*-acting elements involved in ASR-responsive gene expression. ASR can be used to improve crops and economically important plants against various environmental stresses.

1. INTRODUCTION

Pollen development is a postmeiotic process that produces immature pollen grains from microspores. The maturation process can be dissected into various stages. During microspore mitosis, the pollen first develops a large vacuole that mediates nuclear migration to produce polarized cells. Concomitantly, starch begins to accumulate and the vacuolar volume decreases in the maturing pollen. Continued intrinsic polarization of the pollen grains must be maintained before germination. During the final stage of maturation, pollen grains may exhibit developmental arrest, which involves programmed dehydration (Franchi et al., 2011). This dehydration is required for maximum maintenance of pollen viability against various environmental stresses (Twell, 2002). Once fully mature, pollen grains are released from the dehiscent anther for dispersal.

As a consequence of dehydration, the water content of dried pollen becomes severely limited. Aside from dehydration, the induced oxidative stress accumulates reactive oxygen species (ROS) that further damage membrane lipids, proteins, and nucleic acids (Mittler et al., 2004). Thus, the formation of protective molecules during the final stage of pollen maturation is important in ensuring that the pollen withstands drying. The pollen that

withstands drying during maturation becomes desiccation tolerant. Such pollen was recently termed "orthodox" by Pacini et al. (2006). The concept of orthodox pollen is opposed to so-called recalcitrant pollen, which is highly susceptible to desiccation damage because it does not undergo maturation drying and is shed with relatively high moisture content (Franchi et al., 2011). To ensure the survival of orthodox pollen in unfavorable environments, mechanisms associated with desiccation tolerance involve an array of metabolic shutoff genes during developmental arrest and the expression of new genes that encode protective molecules under desiccated conditions (Franchi et al., 2011). A sizable number of protective molecules, such as proline, sucrose, oligosaccharides, and amphipathic molecules, have been identified in desiccated pollen (Lehmann et al., 2010; Pacini et al., 2006; Schwacke et al., 1999). In particular, the well-known dehydrins, a type of late embryogenesis abundant (LEA) proteins initially identified in seeds, were detected in mature pollen grains (Mogami et al., 2002; Wolkers et al., 2001). LEA acts as an osmoprotectant that possesses chaperone-like properties to protect enzymatic activity and prevent protein aggregation resulting from water deficiency (Goyal et al., 2005; Reyes et al., 2005). The pollen LLA23 (a lily ASR) protein has also been found only in the maturation drying stage (Huang et al., 2000; Wang et al., 1996, 1998). Although LLA23 is pollen-specific, the organ or tissue specificity of ASR proteins is diverse in other plant species (Cakir et al., 2003; Liu et al., 2010).

2. DESICCATION-ASSOCIATED ABSCISIC ACID (ABA) SIGNALING IN POLLEN GRAINS

2.1. Gene Expression Profiles during Pollen Maturation

Four studies reported the functional classification of transcripts and proteins found in *Arabidopsis* pollen using a GeneChip array and proteomic analysis (Becker et al., 2003; Holmes-Davis et al., 2005; Honys and Twell, 2003; Pina et al., 2005). These studies provide valuable insights into the complexity of gene expression in mature pollen. The occurrence of various growing stages is supported by a number of earlier studies that indicated the presence of different phases of RNA/protein synthesis during pollen development (Schrauwen et al., 1990). However, information regarding the association of gene expression with maturation drying is rather limited.

To characterize the gene expression profiles at the phase of maturation drying, a subtractive cDNA library was prepared to identify the genes associated with desiccation during anther development (Hsu et al., 2007).

Thirty-four late genes (if LLA23 is taken into account as a control) were identified from lily maturing anthers. Some of the identified desiccation-associated genes were identical or similar to those cold-storage-induced genes identified by Wang et al. (2004). These identified sequences are categorized into several subgroups based on the known functions of gene products and ontology annotations derived from similarities. It includes proteins associated with membrane/cell wall synthesis, regulators/signaling, transporter, cytoskeleton-associated proteins, and so on (Table 2.1). Among these functional categories, the genes involved in membrane/cell wall synthesis represent the largest gene subgroup, which suggests that they are most preferentially expressed in mature anther/pollen. This observation is in agreement with the huge increase in cell wall proteins when the number of different genes expressed is changed into relative expression levels in *Arabidopsis* pollen (Honys and Twell, 2003). Pollen tube elongation is associated with the dynamic changes in the destruction and reconstruction of the cell wall structure and continuous cell–cell interactions in the pistil. Therefore, the genes that encode regulator/signaling and transporter proteins unsurprisingly represent the second largest subgroup of products in mature anther/pollen. An apparent investment in such genes involved in cell wall synthesis, cytoskeleton, and signaling pathways meets the functional requirements needed for pollen germination and tube growth.

During the desiccation of developing anthers, pollen genes are established through three distinct signaling pathways (Fig. 2.1). According to Hsu et al. (2007), signaling pathway I is for the group of genes induced by dehydration, either from environmental water stress or from natural desiccation within the anther. These dehydration-inducible genes require ABA for signaling. About half of the identified genes follow pathway I signaling, among which *LLA23* and *LLP-Rop1* (Rho GTPase of plants) are the two representatives. Expression of *LLA23* is upregulated by ABA upon desiccation, a part of the developmental program that occurs naturally in the anther; thus, its transcript and protein abundantly accumulate in dried pollen (Huang et al., 2000; Wang et al., 1998). The *LLP-Rop1* gene, on the other hand, is downregulated by ABA upon desiccation, whereas the gene is upregulated by other developmental cues. Considering *LLP-Rop1* is not pollen-specific, it is not included in the list in Table 2.1. This unique desiccation-associated ABA signal transduction pathway has recently been reported, through which the *Rop* gene is regulated during pollen maturation (Hsu et al., 2010). However, the lily *Asr* associated with ABA signaling upon desiccation is the focus of this study.

Table 2.1 Identification of subtracted cDNAs at the phase of desiccation during anther/pollen development

Clone no.	Name (Accession no.)	Predicted function	Identity %/ similarity %	Best BLASTX match in database (Accession no.)	E value
Proteins associated with membrane/cell wall synthesis					
9	Xyloglucan endotransglycosylase (EF026010)	Cell wall biosynthesis	64/75 (191)*	*Arabidopsis thaliana* (AAB18365)	2e-69
19	Alpha/beta hydrolase (EF026012)	Hydrolytic enzyme	61/74 (235)	*Oryza sativa* (BAD68323)	3e-69
21	Peroxidase ATP14a homolog (EF026041)	Involved in oxidative stress	52/70 (61)	*A. thaliana* (BAB08340)	4e-10
29	Glycosyltransferase family-like protein (EF026013)	Involved in secondary metabolites	88/97 (67)	*O. sativa* (XP_483148)	1e-29
30	Serine protease (EF026014)	Associated with proteolysis	52/63 (231)	*Lycopersicon esculentum* (CAA07250)	4e-49
36	Pectinmethylesterase inhibitor (EF026015)	Cell wall modification	25/44 (152)	*Actinidia deliciosa* (BAC54965)	9e-05
37	Pectinesterase (EF026016)	Cell wall modification	60/78 (161)	*A. thaliana* (NP_188331)	2e-58
52	Pectate lyase (EF026017)	Cell wall modification	100/100 (244)	*Lilium longiflorum* (CAA78976)	3e-122
59	Cell wall invertase 3 (EF026018)	Hydrolysis of sucrose	63/75 (79)	*O. sativa* (AAV28803)	2e-22
61	Polygalacturonase (EF026019)	Cell wall modification	40/57 (71)	*A. thaliana* (CAA11160)	8e-06
62	Putative phospholipids transfer protein (EF026020)	Biogenesis and renewal of membrane	34/55 (116)	*O. sativa* (XP_464027)	6e-09
72	Extensin-like protein (EF026021)	Structural cell wall protein	33/39 (156)	*Zea mays* (AAD55980)	8e-13

Continued

Table 2.1 Identification of subtracted cDNAs at the phase of desiccation during anther/pollen development—cont'd

Clone no.	Name (Accession no.)	Predicted function	Identity %/ similarity %	Best BLASTX match in database (Accession no.)	E value
Regulator proteins/signaling					
39	ATP-dependent Clp protease regulatory subunit ClpX (EF026028)	Chaperone	94/97 (109)	*A. thaliana* (AAB88706)	1e-51
50	Calcium-dependent protein kinase (EF026029)	Ca^{2+}-mediated signal transduction	86/94 (159)	*N. tabacum* (AAX81331)	1e-73
56	Serine/threonine protein phosphatase (EF026030)	Regulator of ABA signaling	93/100 (80)	*O. sativa* (BAD67848)	4e-41
58	Ring zinc finger protein (EF026031)	Transcription factor	71/80 (76)	*O. sativa* (XP_479841)	1e-26
69	Putative SF16 protein (EF026032)	Predicted nucleic acid-binding protein	43/56 (123)	*O. sativa* (BAD73780)	6e-14
71	Retrotransposon protein (EF026027)	Regulation of gene expression	69/85 (75)	*O. sativa* (ABA95643)	2e-23
Transporter proteins					
20	Putative ABC transporter (EF026034)	Predicted metal resistance protein	68/84 (32)	*A. thaliana* (BAC42008)	7e-05
28	Putative glutathione transporter (EF026035)	Involved in oxidative stress	61/75 (145)	*O. sativa* (XP_483104)	2e-45
54	Histidine amino acid transporter (EF026036)	Nitrogen transport	84/89 (66)	*O. sativa* (CAD89802)	4e-24
57	Plasma membrane H^+ATPase (EF026037)	Electrochemical gradient	95/98 (191)	*L. longiflorum* (AAK31799)	1e-100

64	Monosaccharide transporter (EF026038)	Sugar transporter	80/89 (205)	*O. sativa* (AAQ24872)	5e-84
90	Isp4-like protein (EF026039)	Oligopeptide transport	65/78 (78)	*A. thaliana* (CAB79514)	2e-23
Cytoskeleton and associated proteins					
13	IFA binding protein (EF026011)	Cytoskeletal binding protein	40/61 (415)	*O. sativa* (BAD52954)	4e-07
87	Beta-5 tubulin (EF026040)	Cytoskeletal protein	100/100 (23)	*Z. mays* (CAA52720)	5e-06
Others and unknown†					
82	Haemolysin-III related family protein (EF026033)	Unknown	75/87 (386)	*O. sativa* (ABF94811)	5e-163
89	Nodulin-like protein (EF026042)	Unknown	55/74 (168)	*A. thaliana* (AAC28987)	2e-41

⋆Parentheses indicate the number of amino acids of each clone used for the comparison.
†No significant match was found for clone no. 12 (EF026022), 14 (EF026023), 24 (EF026024), 40 (EF026025), and 73 (EF026026).
Table 2.1 is cited *from Hsu et al. (2007) with kind permission from Springer Science and Business Media.*

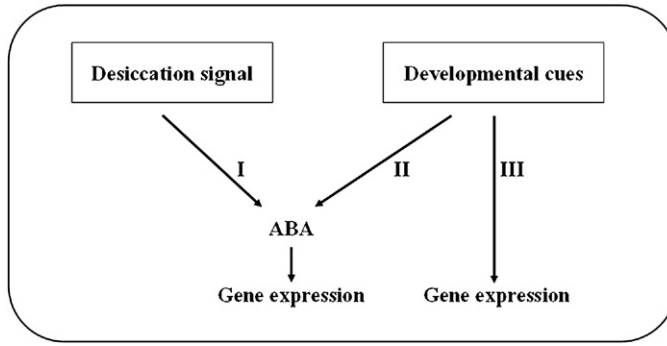

Figure 2.1 Schematic diagram of the proposed signaling pathways from the perception of dehydration via abiotic stress and developmental cues to gene expression in lily pollen. At least three signal transduction pathways exist: pathway I is for the group of genes induced by environmental dehydration stress and is ABA-dependent. The other two pathways are for the dehydration-irrelevant genes triggered by developmental desiccation and other cues in the anther, through which pathway II is ABA-dependent and pathway III is ABA-independent . *(From Hsu et al. (2007) with permission from Springer Science and Business Media).*

The dehydration-inducible genes in pathway I require ABA for signaling. However, the induction of dehydration-induced genes can be independent of ABA (Shinozaki and Yamaguchi-Shinozaki, 1997). Pathway II includes dehydration-irrelevant genes, and their induction is ABA-dependent. Pathway III of signal transduction is for dehydration-irrelevant genes, but its signaling is ABA-independent. For instance, polygalacturonase accumulates in mature pollen through developmental cues, but it is not induced by desiccation and exogenous ABA (Chiang et al., 2006; Hsu et al., 2007). Although it cannot be induced by dehydration and ABA, its expression is influenced by environmental stresses such as heat, high CO_2, low temperature, and ethylene (Dopico et al., 1993; Hiwasa et al., 2003; Kagan-Zur et al., 1995; Rothan et al., 1997). The genes in pathways II and III, triggered by developmental cues other than desiccation in the anther, are intriguing because little is known about the regulation of such genes.

2.2. Proteins Encoded by Desiccation-Associated Genes in Pollen

Pollen contains large amounts of presynthesized mRNAs and proteins (Mascarenhas, 1993; Taylor and Hepler, 1997; Twell, 2002). However, the presence of presynthesized mRNAs and proteins needed for pollen germination and tube growth should adapt to the highly dehydrated state of the pollen. Of the 34 identified proteins encoded by desiccation-associated

genes in pollen (Hsu et al., 2007), almost half (15) exhibit high-average hydrophilicity based on hydropathy profile analysis (Kyte and Doolittle, 1982). The rest of proteins, however, should be protected by those osmoprotective molecules to maintain their biological functions.

Shinozaki et al. (2003) classified the products of drought-inducible genes into two groups. The first group includes proteins directly involved in plant survival under stressful conditions. Several of the 34 desiccation-associated proteins listed in Table 2.1 were classified into the first group after examination. For instance, various proteases, such as alpha/beta hydrolase (clone 19), serine protease (clone 30), pectinesterase (clone 37), and pectate lyase (clone 52), may be involved in stress defense. The putative SF16 protein (clone 69) may be involved in the stability and maturation of pollen mRNAs (Dudareva et al., 1994). LLA23 acts as a chaperone and a regulator upon drying (Yang et al., 2005, 2008). The peroxidase ATP14a homolog (clone 21) and putative glutathione transporter (clone 28) may be involved in oxidative detoxification. Moreover, several transporters, such as histidine amino acid transporter (clone 54), monosaccharide transporter (clone 64), and Isp4-like protein (clone 90), exist in the dried pollen for the transportation of sugar and amino acids. The plasma membrane H^+ ATPase (clone 57) may be associated with ion homoeostasis. In addition, the HVA22 homolog, an LEA protein, is also included in this group, although it begins to accumulate during the early maturation stage and continues to accumulate until the full maturation of the anther (Hsu et al., 2007).

The second group is composed of regulatory proteins that include transcription factors and other signaling molecules, such as ATP-dependent Clp protease regulatory subunit ClpX (clone 39), calcium-dependent protein kinase (clone 50), serine/threonine protein phosphatase (clone 56), ring zinc finger protein (clone 58), a retrotransposon protein (clone 71) and LLA23 (Table 2.1). Taken together, the division into two groups of drought-inducible genes proposed by Shinozaki et al. (2003) may be applied to the desiccation-associated genes in dried pollen as well.

3. ASR

3.1. Gene Expression and Organization of the *Asr* Genes

3.1.1. Distribution and Spatial and Temporal Expression

Asr is a stress-inducible gene that is reportedly exclusively to plants. Since Iusem et al. (1993) described the first *Asr* gene from tomato, *Asr* genes have been found in various species of dicotyledonous and monocotyledonous

plants, including potato (Frankel et al., 2007; Silhavy et al., 1995), pomelo (Canel et al., 1995), loblolly pine (Chang et al., 1996), apricot (Mbeguie-A-Mbeguie et al., 1997), maize (Riccardi et al., 1998), lily (Huang et al., 2000; Wang et al., 1998), rice (Kim et al., 2009; Vaidyanathan et al., 1999; Yang et al., 2004), *Cucumis melo* (Hong et al., 2002), grape (Cakir et al., 2003), *Ginkgo biloba* (Shen et al., 2005), plantain (Liu et al., 2010), and strawberry (Chen et al., 2011). However, these genes are not present in *Arabidopsis*. Recently, an *Asr* gene, *SbASR*-1, was identified in an extreme halophyte *Salicornia brachiata* that has a unique genetic makeup with well-developed adaptation mechanism to survive in a saline condition (Jha et al., 2012).

Among various species, *ASR* genes are expressed in different organs, such as the fruits of tomato, pomelo, apricot, melon, grape, and strawberry (Canel et al., 1995; Chen et al., 2011; Iusem et al., 1993; Mbeguie-A-Mbeguie et al., 1997); the roots, leaves, and seedlings of tomato, rice, pine, *Ginkgo biloba*, and maize (Amitai-Zeigerson et al., 1994; Cakir et al., 2003; Chang et al., 1996; Hong et al., 2002; Liu et al., 2010; Riccardi et al., 1998; Shen et al., 2005; Vaidyanathan et al., 1999; Yang et al., 2004); the leaves, stems, and tubers of potato (Frankel et al., 2007; Silhavy et al., 1995); developing tomato seeds (Maskin et al., 2008); and pollen of lily (Huang et al., 2000; Wang et al., 1998). Although the *Asr* gene may be expressed in different organs under different conditions, the lily *Asr* is pollen-specific and is only expressed during the maturation drying stage of anther/pollen development (Huang et al., 2000; Wang et al., 1998).

3.1.2. Gene Polymorphism and Adaptation

The lily *Asr* gene structure consists of two exons separated by one intron (Yang et al., 2008). The result is consistent with four *mAsr* genes and other *Asr* sequences from various species (Henry et al., 2011). All *Asr* genes typically have two exons, one intron structure, and their characteristic ABA/water-deficit stress (WDS) domain. In most species, *Asr* genes belong to a small gene family. The number of plant *Asr* genes available in plant genome varies from one in grape (Cakir et al., 2003), at least four in banana (Henry et al., 2011), six in rice (Philippe et al., 2010), nine in maize (Virlouvet et al., 2011) and four in pine and tomato (Chang et al., 1996; Frankel et al., 2007). Recently, Fischer et al. (2011) discovered a new member of the gene family, *Asr5*, in tomato. The comparison of the sequences from different species suggests that family members from a single species are more closely related to each other than to *Asr* members from other species (Carrari et al., 2004; Philippe et al., 2010). This observation suggests that *Asr* genes may originate

from a combination between tandem duplication and whole genome duplication events, and that their differential regulation under water stress probably involves subfunctionalization (Philippe et al., 2010).

The polymorphism of *Asr* genes can also be extended to allelic diversity. The grape *VvMSA* gene exhibits allelic polymorphism (Saumonneau et al., 2012) based on the BLAST analysis of the *VvMSA* gene sequence against the proteome 8× deduced from the Pinot noir genome (Jaillon et al., 2007). The *mAsr3* and *OsAsr3* genes exhibit the most sequence variation among the cultivars tested in banana and rice (Henry et al., 2011; Philippe et al., 2010). *OsAsr3* is the most divergent of the rice *Asr* genes and exhibits overall neutral selection at the species level, but directional selection in the japonica subgroup found in tropical regions (Philippe et al., 2010). Similar to *mAsr3*, the presence of polymorphic variants of the water stress-induced *Asr2* gene of tomatoes undergoes adaptive changes to possibly adapt to arid habitats (Frankel et al., 2003; Giombini et al., 2009). By contrast, Fischer et al. (2011) suggested that the tomato *Asr4* gene, but not *Asr2*, shows patterns consistent with local adaptation living in an extremely dry environment, whereas *Asr1* has evolved under strong purifying selection.

3.2. ASR Protein

3.2.1. Structural Features

The lily LLA23 protein is rich in Glx and Gly, a characteristic of heat-stable proteins (Wang et al., 1996). The predicted molecular mass of LLA23 is around 16 kDa; however, the protein ran at 23 kDa when fractionated under SDS-polyacrylamide gel electrophoresis (Wang et al., 1996). This high apparent molecular mass is caused by a highly hydrophilic property, which is often characteristic of intrinsically unstructured proteins (Tompa, 2002). The protein contains two main highly conserved regions, a small N-terminal consensus region containing a stretch of six His residues and a large consensus region at the C-terminus. The six His residues at the N-terminal consensus may bind with zinc ions. Goldgur et al. (2007) suggested that a pair of zinc ions tightly bound with ASR1 induces homodimer formation. A dimeric protein structure is a commonly observed motif among DNA-binding proteins (Burley and Kamada, 2002). In the C-terminal consensus sequence, it possesses two ABA/WDS signature sequences (Canel et al., 1995; Padmanabhan et al., 1997) and a putative nuclear localization signal (NLS) sequence at the end of the molecule (Wang et al., 2005). The bipartite NLS motif of the lily ASR, **KK**TL**KK**ENEEVEG**KK**, has two functional clusters of basic amino acids separated by a spacer of several nonconserved residues.

Mutations in the NLS motif of LLA23 contain two defined regions, either of which is necessary for partial nuclear targeting but both are required for complete nuclear localization (Wang et al., 2005). The putative NLS is also conserved in other members of ASR proteins both in dicots and in monocots (Liu et al., 2010). The reported presence of putative bipartite NLSs in a high proportion of plant b-ZIP proteins (Raikhel, 1992; Varagona et al., 1992) suggests that the bipartite structure may be the most prevalent NLS configuration in plants across a spectrum of divergent nuclear proteins.

3.2.2. Intrinsically Unstructured Protein

The lily ASR protein is stable at 90 °C for 10 min (Wang et al., 1996) and the boiling-soluble feature is due to its flexible conformation in solution. The protein contains relatively poor order-promoting amino acid residues and enriched disorder-promoting amino acid residues that result in the intrinsically unstructured conformation of the polypeptide (Dai et al., 2011; Goldgur et al., 2007). Intrinsically unstructured proteins have been found to play crucial roles despite the lack of certain high-ordered structures (Dyson and Wright, 2005). For example, the unstructured form of ASR1 has chaperone-like activity that helps stabilize macromolecules against denaturation caused by heat and freeze–thaw cycles (Hsu et al., 2011; Konrad and Bar-Zvi, 2008). The hydrophilic and boiling-soluble ASR protein may function as an intracellular water retainer for preventing local dehydration during stress (Dai et al., 2011; Yang et al., 2005). Furthermore, the protective activity of ASR1 is synergistic with glycine-betaine, which accumulates under unfavorable environmental conditions (Konrad and Bar-Zvi, 2008). In addition, ASR can fold into an ordered structure and form a homodimer upon binding with zinc ions (Goldgur et al., 2007).

Uversky et al. (2000) indicated that most natively unfolded proteins have low-mean hydrophobicity, high net charge, and low molecular weight, with less than 150 amino acid residues. For example, LEA proteins have a relatively high composition of hydrophilic amino acids, most of which are disordered under native conditions (Battaglia et al., 2008; Mouillon et al., 2008). ASR is another example that fits these criteria. As a hydrophilin, Battaglia et al. (2008) classified ASR as a Group 7 LEA protein.

3.2.3. Cellular Localization

Subcellular fractionation experiments and immunodetection in tomato fruit chromatin fractions suggest that tomato ASR1 is localized in the nucleus (Iusem et al., 1993). Although ASR easily passes through the nuclear pore

because of its small size, it can be actively transported using the NLS sequence in the molecule. As shown in Fig. 2.2, the GFP-LLA23 fusion protein is localized predominantly in the nucleus of leaf cells, whereas GFP alone was distributed in the cytoplasm. By contrast, deletion of the NLS sequence (mutGFP-ΔLLA23) results in the cytoplasmic localization of the fusion protein (Fig. 2.2). Immunogold localization confirmed that the protein is located in the nucleus of generative and vegetative cells in pollen grains (Wang et al., 2005). The localization of ASR in the nucleus is also supported by the fact that these nuclear basic proteins bind DNA, as demonstrated by DNA gel blot, filter, and chromatin binding for tomato ASR1 via atomic force microscopy (Maskin et al., 2007) and in vivo chromatin immunoprecipitation assay (Shkolnik and Bar-Zvi, 2008). ASR1 forms homodimers with a zinc-dependent DNA-binding activity (Kalifa et al., 2004a; Maskin et al., 2007) or, as discovered in a grape ASR protein, heterodimers with

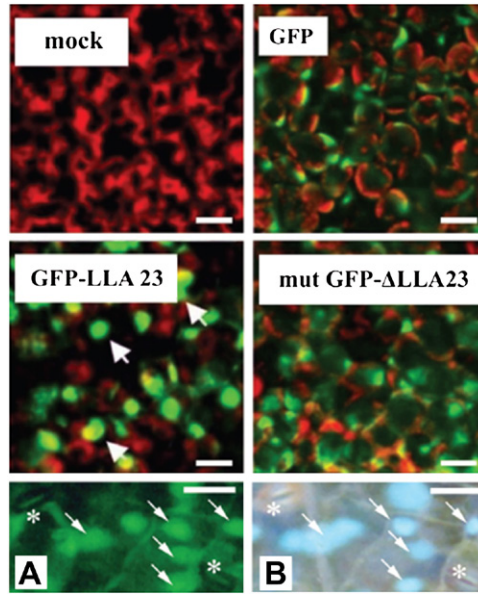

Figure 2.2 Representative images of the subcellular localization of a mock control, green fluorescent protein (GFP), GFP-LLA23 and mutant GFP-ΔLLA23 constructs expressed in bamboo mosaic potexvirus (BaMV)-infected leaves of *Chenopodium quinoa*. The images were obtained 7 days after infection on *C. quinoa* leaves. The locations of GFP-LLA23 (A) were exactly the same as those stained with 4′, 6-diamidino-2-phenylindole (DAPI) (B). Arrows indicate GFP in the nucleus. Asterisks indicate the corresponding guard cells. Scale bar represents 20 mm. *(Modified from Wang et al. (2005) with permission from Blackwell Munksgaard Societas Physiologiae Plantarium Scandinavica).* For color version of this figure, the reader is referred to the online version of this book.

drought response element binding (DREB) proteins (Saumonneau et al., 2008). Shkolnik and Bar-Zvi (2008) have further demonstrated the tomato ASR1-bound coupling element 1 (CE1) of ABI4 promoter competing with ABI4, which produced an ABA insensitive phenotype of ASR1-over-expressing plants in *Arabidopsis*.

In addition to nuclear localization, substantial amounts of ASR still remain in the cytoplasm. Kalifa et al. (2004a) indicated that two-thirds of the total ASR1 protein is detected in the cytoplasm using indirect immunofluorescence analysis. In pollen grains, ASR is located both in the cytoplasm and in the nucleus of generative and vegetative cells, which has been confirmed using the immunogold labeling of LLA23 (Huang et al., 2000; Wang et al., 2005). Embryogenic calli of transgenic rice carrying an ASR5–GFP fusion revealed that ASR5 was localized in both the nucleus and cytoplasm (Arenhart et al., 2012). Aside from ASR, several nuclear proteins are also found in the cytoplasm (Houde et al., 1995; Yoneda, 2000). The ASR protein remaining in the cytoplasm is quite intriguing. Although the actual mechanism by which the protein is retained in the cytoplasm is unknown, it may perhaps involve either the masking of NLS or protein–protein interactions that serve to anchor the regulatory protein in the cytoplasmic compartment (Beaudoin and Labbé, 2006).

3.2.4. Nucleocytoplasmic Transport of ASR

In addition to the small molecules that diffuse passively through nuclear pores along their concentration gradient, large molecules can be actively transported using the NLS sequence recognized by receptors that dock with their cargo at the nuclear pore (Jans et al., 2000; Yoneda, 2000). The proteins in the nucleus or in the cytoplasm are at least partly regulated by dehydration. Dehydration induces the translocation of LLA23 from the cytoplasm into the nucleus during pollen maturation (Yang et al., 2008). Multiple nucleoplasmic transport pathways have been suggested (Yoneda, 2000). The nucleocytoplasmic transport of proteins is regulated by the masking/unmasking reactions of NLSs through their binding proteins (Jans et al., 2000; Yoneda, 2000). For example, a transcription factor, Cuf1, is localized in the cytoplasm via the masking of its NLS by intramolecular conformational changes in the induced metalation of Cuf1 in the presence of elevated copper concentrations (Beaudoin and Labbé, 2006). In some cases, the heterodimerization or homodimerization of proteins is involved in the regulation of the nuclear import of proteins. The DNA-bound ASR1 is folded in a dimeric protein structure (Goldgur et al., 2007); however,

whether the dimerization of ASR, if present in the cytoplasm, regulates the translocation of ASR into the nucleus is still unclear. Phosphorylation/dephosphorylation reactions are considered the most common mechanism involved in the regulation of the nucleocytoplasmic transport of proteins (Jans et al., 2000; Yoneda, 2000). The yeast transcription factor SWI5 is located in the cytoplasm when it is phosphorylated near its NLS, whereas it accumulates in the nucleus when dephosphorylated (Moll et al., 1991). For ASR, no phosphorylation site is identified in the sequence based on the motif analysis. Thus, the regulation of nucleocytoplasmic transport of ASR needs further investigation.

3.3. A Dual Function of ASR

Yang et al. (2005) have proposed a dual function of the lily ASR protein being a regulator in the nucleus and an osmoprotectant in the cytoplasm. As an osmoprotectant, the unstructural molecules of ASR in the cytoplasm may act as chaperones stabilizing other proteins and enzymes against denaturation caused by heat, cold and freeze–thaw cycles (Hsu et al. 2011; Konrad and Bar-Zvi, 2008), as a water replacement molecule protecting membrane integrity in a manner similar to sugars (Clegg et al., 1982), and at least in part as a water-holding molecule retaining water in the cell as observed in *Arabidopsis* plant overexpressing LLA23 or MpASR (Dai et al., 2011; Yang et al., 2005). Like LLA23, other LEA proteins have been hypothesized to play a similar manner of protective role under unfavorable environments based on their high-average hydrophilicity (Goyal et al., 2005; Reyes et al., 2008). LEAs can act as protein stabilizers and help the plant cells to retain water during periods of low water availability (Garay-Arroyo et al., 2000; Kovacs et al., 2008). At very low water content and high cytoplasmic viscosity, LEAs and sugars have been proposed to form a tight hydrogen-bonding network together in the dehydrated cytoplasm (Wolkers et al., 2001). Constitutive overexpression of LEAs reported by several studies exhibits a significant increase in their tolerance to dehydration, salt, or other stress conditions (Dalal et al., 2009; Lal et al., 2008; Olvera-Carrillo et al., 2010).

The secondary level of protection comes from the regulatory properties of LLA23 proteins as described above. The expression of *RD29b* and *KIN2* is upregulated in *35S::LLA23* plants, suggesting that LLA23 can modulate stress-responsive ABA signaling (Yang et al., 2005). The massive production of other osmoprotectant molecules greatly enhances the stress tolerance of *35S::LLA23* plants under unfavorable conditions. The osmoprotectants, also known as osmolytes or compatible solutes, are highly hydrophilic

organic molecules that accumulate in cells in response to desiccation and other abiotic stresses. The most common osmoprotectants are betaine, proline and soluble sugars, which can help to maintain the conformation of proteins or act as chaperones to help in the refolding of the proteins that are prone to aggregation or unfolding during cellular dehydration (Pareek et al., 2010). Recent reports have shown that overexpression of transcription factor genes improves stress tolerance (Gao et al., 2011; Xu et al., 2011).

4. TRANSGENIC *35S::LLA23* PLANTS

4.1. Transgenic *35S::LLA23* Plants Exhibit Desiccation-Resistant Phenotype

The coding region of LLA23 fused to the cauliflower mosaic virus 35S promoter was used to transform *Arabidopsis* (ecotype Columbia [Col]) plants and T3 kanamycin-resistant homozygous lines were generated. The *35S::LLA23* plants are less sensitive to ABA and more dehydration-tolerant than wild-type plants. As shown in Fig. 2.3A and B, the growth and development of LLA23-overexpressing plants in soil in the growth chamber without stress appeared normal. When the soil was allowed to dry by withholding water, 48% of both independent *35S::LLA23* lines and 84% of the wild-type plants became wilted 12 days after the withdrawal of water (Fig. 2.3C). The two *35S::LLA23* lines that remained upstanding did not begin to wilt until 16 days after the withdrawal of water (data not shown). When the wilted plants were rewatered afterward, only 5% of the wilted wild-type plants recovered, whereas 50% of both wilted *35S::LLA23* lines survived to maturity (Fig. 2.3D). Corroborating data were obtained when the rate of water loss from detached leaves of wild-type and LLA23-overexpressing plants was compared. The leaves tested were of similar size and age. Leaves of transgenic plants overexpressing the LLA23 protein had lower rates of water loss than that of wild-type plants (Fig. 2.3E). Microscopic

of each genotype and three replicates each). Wilted plants were then rewatered (day 13), and the number of recovered plants (fully regained turgor and resumed growth) was scored after an additional 4 days (day 16) and represented as the percentage of the wilted plants. Open bar, wild type; closed bars, *35S::LLA23C*; gray bars, *35S::LLA23E*. Error bars represent SD. E: Water loss from the leaves of wild-type and *35S::LLA23* plants. Detached leaves of wild-type or LLA23-overexpressing plants were assayed for water loss. F: Micrographs showing the stomatal closure regulation of *35S::LLA23E* and wild-type plants under drought stresses for 12 and 16 days, respectively. All images were taken at the same scale (bar = 30 mm). *(From Yang et al. (2005) with permission from American Society of Plant Physiologists).*

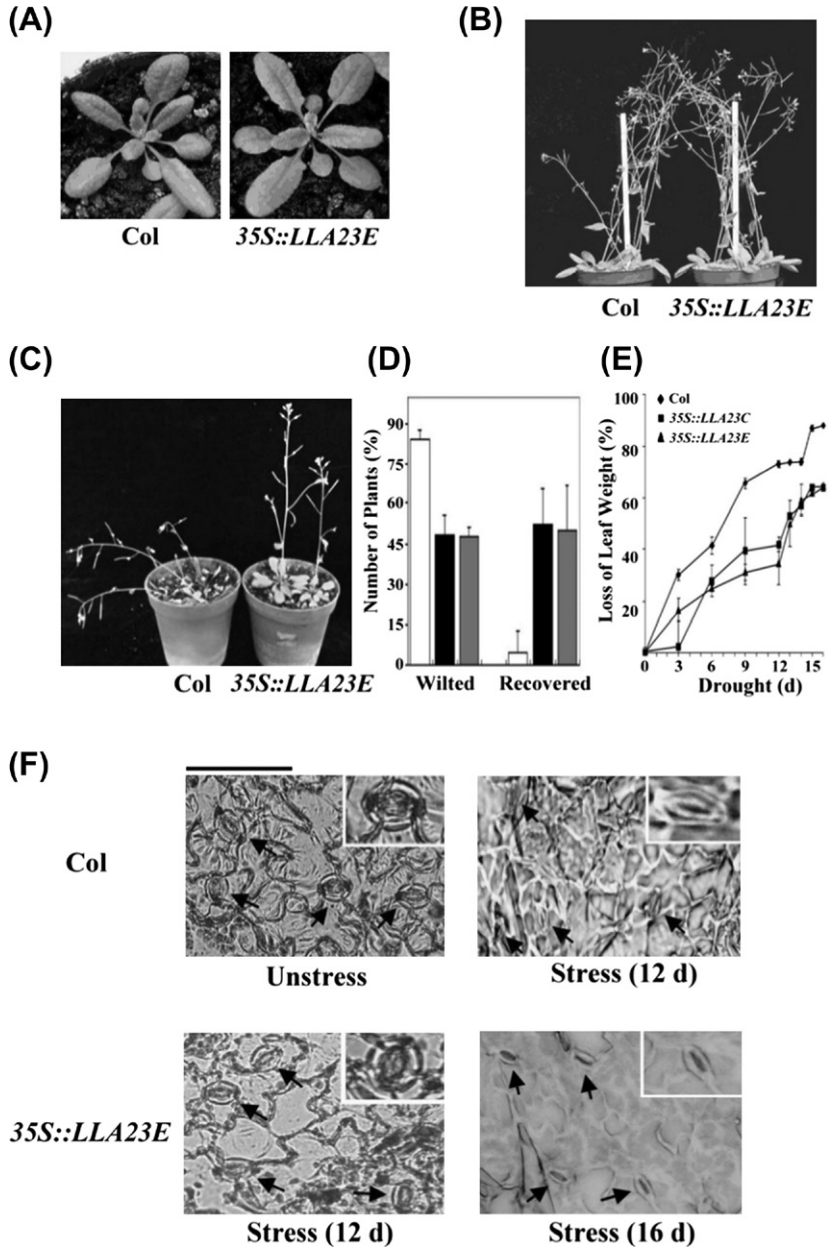

Figure 2.3 ***Drought tolerance of 35S::LLA23 transgenic plants.*** A–C: Wild-type (Col) and *35S::LLA23E* plants were grown on soil for 3 weeks (A), withheld water for 12 days, and then rewatered (C). The photographs were taken 4 days after the rewatering. B is a control of C, without withholding water. D: The number of wilted plants at the end of day 12 was scored and represented as the percentage of the total plants (from 40 plants

examination of stomatal opening of *35S::LLA23* leaves showed that approximately 86% (78/91) of *35S::LLA23E* stomata remained open under drought stresses for 12 days, a percentage close to 96% (74/77) of the wild-type stomata without stress (Fig. 2.3F). The unexpected observation of stomatal opening was further checked by measuring the levels of ABA of both plant types. Correlated with the opening of *35S::LLA23* stomata, the transgenic plants did not appreciably increase their ABA levels upon drought stresses for 12 d, similar to that of the wild-type plants at normal growth conditions (Yang et al., 2005). It clearly indicated that due to the presence of LLA23 proteins, the response to ABA was delayed in the transgenic plants upon water deficit. The LLA23 protein functions as a water-retaining molecule that confers drought resistance on *35S::LLA23* plants based on the drought test of transgenic lines, in which leaves of these plants overexpressing LLA23 protein had lower rates of water loss than wild-type plants (Fig. 2.3E), while *35S::LLA23* stomata remained open upon drought stresses for 12 days. Nevertheless, if these plants were continued to withhold water for additional 4 days, most *35S::LLA23* stomata (87%) became closed (Fig. 2.3F); transgenic plants began to wilt and their ABA levels in leaves markedly increased.

The transpiration rate of transgenic plants was also examined. As shown in Fig. 2.4A, *35S::LLA23* leaves showed pattern of transpiration rate similar to the wild-type leaves upon drought stress despite that 91% (91/100) of *35S::LLA23E* stomata remained open, which is opposite to 83% (88/106) of the closed stomata observed in the wild-type leaves (Fig. 2.4B). It is rational that ABA in the wild-type leaves was apparently produced and let most stomata (88/106) become closed upon drought stress while under the same drought condition, ABA in the transgenic plants did not change, similar to the unstressed wild-type plants and thus most stomata (91/100) remained disclosed. The fact that LLA23-overexpressing plants kept approximately the same transpiration rate as the wild-type plants while their stomata mostly remained open strongly suggests the function of LLA23 as a water-retaining molecule in the *35S::LLA23* leaves. It correlates with the results obtained when the rate of water loss from detached leaves of wild-type and LLA23-overexpressing plants was compared (Fig. 2.3E). The *gin1-3* mutant, however, displayed a much more pronounced transpiration rate because the mutant was unable to produce ABA to which stomata respond and thus, 96% (78/82) of *gin1-3* stomata remained open (Fig. 2.4B). In addition to acting as a water-retaining molecule, the LLA23 protein may protect other macromolecules from losing their biological

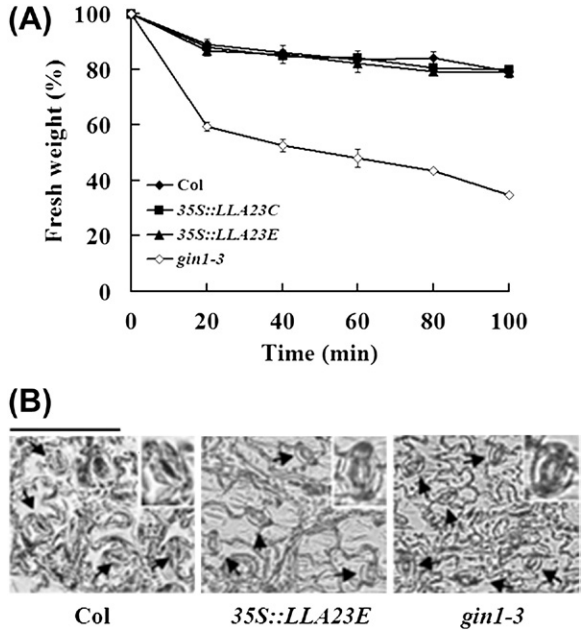

Figure 2.4 ***Analysis of the transpiration rates in 35S::LLA23 transgenic leaves.*** A: The wild-type, *35S::LLA23*, and *gin1-3* mutant plants were grown on soil for 3 weeks. The detached leaves of wild-type, LLA23-overexpressing, or *gin1-3*-mutant plants were placed on the laboratory bench for indicated time intervals at 25 °C before the measurement of weight for transpiration rate analysis. B: Micrographs showing the stomatal closure regulation of *35S::LLA23E*, *gin1-3*, and wild-type plants at the time interval of 100 min, respectively. All images were taken at the same scale (bar = 30 mm).

structure and function in the cytoplasm upon desiccation. The high hydrophilicity and abundance of LLA23 facilitate its protection against the dried condition. We have recently evidenced that the LLA23 protein can significantly protect enzymes from losing their activities under the conditions of cold and freezing stresses (Hsu et al., 2011). It is intriguing that Silhavy et al. (1995) suggested that the potato DS2, a member of ASR, may play a role of protection of the nuclear DNA against desiccation. It is possible that the LLA23 protein might do the same for the DNA in the nucleus of pollen grains. In addition, the unstructured ASR1 monomers in the cytoplasm, act as chaperons, possibly to prevent proteins losing their structure during desiccation (Konrad and Bar-Zvi, 2008). The presence of these proteins in both cytoplasm and nucleoplasm suggests that they are involved in a general protective role, but might display a distinct function in each compartment.

4.2. ASR-Inducible Genes in *35S::LLA23* Plants

A microarray analysis of an LLA23-overexpressing transgenic line was conducted to gain a more comprehensive view of LLA23-regulated gene expression. Homozygous *35S::LLA23E*, which has higher LLA23 expression levels, was selected for the analysis in parallel with wild-type *Arabidopsis* (Col) plants. The genotype-specific signal intensity for each of the 22,810 genes on the arrays was determined and analyzed with a *t*-test for significant differences ($P < 0.05$). Only the genes with high significance ($P < 0.05$) and fold change greater than 2 were selected. Thus, 206 genes with expression ratios greater than twofold induction of expression and 204 genes with expression ratios greater than twofold repression of expression in the LLA23-overexpressing plants were identified (Yang et al., 2008). All these responsive genes are proposed to be modulated by LLA23. The upregulated and downregulated genes (101/12) with two and halffold induction or repression in LLA23-overexpressing plants are listed in Table 2.2.

4.2.1. Classification of LLA23-Inducible Genes

The 101 LLA23-inducible gene products can be classified into two groups. The first group includes functional proteins or proteins that probably function in stress tolerance, including detoxification enzymes, LEAs, cold-regulated proteins, transporters, water channel proteins, and cell wall-related and defense-related proteins (Table 2.2). Detoxification enzymes such as peroxidase (At3g01190) and glutathione S-transferase (At1g49860) are considered to be involved in the protection of cells from active oxygen radicals. LEAs have been shown to be involved in protecting macromolecules, such as enzymes and lipids (Hanin et al., 2011; Huang et al., 2012). Peptide transporters, carrier and water channel proteins are involved in the transport of ions, water and proteins through plasma membranes and tonoplast to adjust the osmotic pressure. Cell wall-related enzymes and extensin-like protein are involved in structural fortification and remodeling of cell wall upon exposure to pathogens and abiotic stresses (Merkouropoulos and Shirsat, 2003). Among defense-related proteins, nonspecific lipid-transfer proteins may exhibit antimicrobial activity upon various stresses (Sun et al., 2008). The gene encoding dioxygenase-like protein (At1g14120) is responsive to pathogen and stress (Umate, 2011; Xu et al., 2010). In addition, pinoresinol reductase (At4g13660) may be involved in the production of polyphenolic substances (antioxidants) (Nakatsubo et al., 2008). Importantly, several ASR-induced enzymes such as 3-deoxy-D-manno-octulosonic acid transferase (At5g03770), 4-alpha-glucanotransferase (At5g64860), indole

Table 2.2 Number of LLA23-inducible or -repressive genes* involved in different functional groups in *35S:LLA23* plants

Functional category	Gene number	Representative gene names
Inducible genes (101)		
Transcription factor	7	bZIP transcription factor (At1g68880), DREB2B transcription factor (At3g11020), GRAS family transcription factor (At5g41920), putative DNA-binding protein (At1g74500), RGA1-like protein (At3g03450), †pseudo-response regulator 3 (At5g60100), #helix–loop–helix DNA-binding protein (At5g53210)
Signaling	9	Patatin-related phospholipase A (At4g37070), lysophospholipase (At3g15650), protein phosphatase 2C (At1g16220), WD-repeat protein-like protein (At4g33260), receptor protein kinase (At4g36180), serine/threonine kinase (At1g54510), similarity to protein kinase (At1g51940), †protein kinase (At2g23030), †receptor-like protein kinase (At2g28960)
Detoxification	3	Glutathione S-transferase (At1g49860), selenium-binding protein (At3g23800), †peroxidase (At3g01190)
LEA protein	2	Late embryonic abundant protein (At5g60520), †late embryogenesis protein (At1g54890)
Cold-related	2	Putative cold-regulated protein (At4g38410), †stress-responsive protein homolog (At4g30660)
Transporter	12	ABC transporter (At2g39350), amino acid transporter (At5g23810), bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein (At5g46900), uclacyanin I (At2g32300), zinc transporter (At3g58810), Zrt- and Irt-related protein (ZIP) family (At2g32270), †metal-binding protein (At1g22990), †nodulin MtN21/EamA-like transporter (At4g28040), †peptide transporter (At4g21680), †phosphatidylinositol transfer protein (At3g24840), †ABC transporter (At1g15210), †mitochondrial carrier protein (At2g37890)
Water channel protein	1	#Putative aquaporin (tonoplast intrinsic protein) (At2g25810)
Cell wall-related	8	Putative beta-glucosidase (At2g44460), EXLA1 (expansin-like A1) (At3g45970), xyloglucan endo-1,4-beta-D-glucanase (At4g25820), two †beta-glucosidase protein (At1g61820, At3g60140), #extensin-like protein (At5g35190), #xylan endohydrolase (At1g58370), #acyltransferase (At5g23940)

Continued

Table 2.2 Number of LLA23-inducible or -repressive genes* involved in different functional groups in *35S:LLA23* plants —cont'd

Functional category	Gene number	Representative gene names
Defense-related	6	Disease resistance-responsive (dirigent protein) (At1g64160), germin-like protein GLP6 (At5g39110), mildew resistance locus o 10 (At5g65970), nonspecific lipid-transfer protein (At3g08770), †pinoresinol reductase (At4g13660), #dioxygenase-like protein (At1g14120)
Carbohydrate metabolism	6	3-deoxy-D-manno-octulosonic acid transferase (At5g03770), indole glucosinolate O-methyltransferase 2 (At1g21120), similar to glycosyltransferase (At1g02720), †4-alpha-glucanotransferase (At5g64860), two #FAD-binding berberine family protein (At1g30720, At5g44380)
Fatty acid metabolism	1	Fatty acid reductase 1 (At5g22500)
Secondary metabolism	1	†Oxysterol-binding protein-like (At5g57240)
Amino acid and protein metabolism	3	Branched-chain amino acid aminotransferase (At1g50110), translation initiation factor 3 (IF-3) protein (At1g34360), †cysteine proteinase (At3g19390)
Cytoskeleton	2	Actin depolymerizing factor (At1g01750), similar to kinesin-like protein (At3g23670)
DNA, nucleus	1	Chromatin assembly factor-2 (FAS2) (At5g64630)
Auxin-related	1	SAUR-like auxin-responsive protein (At4g31320)
Jasmonate-related	1	Similar to jasmonate-inducible protein (At2g25980)
Pollen allergen	1	#Pollen allergen-like protein SAH7 protein (At5g10130)
Unclassified	34	†Pentatricopeptide repeat (PPR) superfamily protein (At5g02860), 12 hypothetical proteins (At4g17800, At1g08430, At1g63240, At1g09220, At3g48510, At3g02680, At1g29270, At1g26900, At4g31270, At4g17240, At3g59370, At1g10990), putative protein (At3g50140), two putative predicted proteins (At5g14730, At4g21070), six unknown proteins (At2g24980, At2g19800, At1g19530, At3g02240, At1g10800, At5g67480), three †hypothetical proteins (At3g13674, At3g15140, At3g12700), †putative predicted protein (At5g19340), two †unknown proteins (At2g20480, At3g22570), two #expressed proteins (At1g53345, At4g37295), two #hypothetical proteins (At1g75150, At2g45900), #putative predicted protein (At4g24110), ‡hypothetical protein (At4g27510)

Repressive genes (12)

Transcription factor	1	[#]WRKY-type DNA-binding protein (At2g38470)
Heat shock protein	1	Heat shock protein 101 (At1g74310)
Transporter	2	Mitochondrial dicarboxylate carrier (At4g24570), [†]peptide transporter (At1g64500)
Defense-related	2	Pathogenesis-related PR-1-like protein (At2g14610), [#]nudix hydrolase homolog 21 (NUDT21) (At1g73540)
Photosynthesis	2	5-adenylylsulfate reductase (At4g04610), [§]PRH26 protein (paps reductase homolog 26) (At4g21990)
Unclassified	4	Two hypothetical proteins (At4g29780, At1g04770), putative protein (At4g23810), [‡]unknown protein (At1g04010)

[★]Inducible and repressive genes with expression ratios greater than two and halffold in LLA23-overexpressing plants.
[†]Induced or repressed by all three stresses.
[‡]Highly induced or repressed genes by ASR only.
[#]Highly induced or repressed genes by both ASR and glucose.
[§]Highly repressed genes by both ASR and drought.

glucosinolate O-methyltransferase 2 (At1g21120) and FAD-binding berberine family proteins (At1g30720 and At5g44380) may participate in various metabolic processes of a variety of carbohydrates and sucrose (Belunis and Raetz, 1992; Hemm et al., 2003; Lee et al., 2005; Lütken et al., 2010). Kinesin-like protein (At3g23670) may be involved in the microtubule control of cellulose microfibril order (Zhong et al., 2002). These LLA23-regulated genes are consistent with the reported grape ASR whose function is involved in sugar metabolism and a common transduction pathway of sugar and ABA signaling (Cakir et al., 2003; Frankel et al., 2007; Saumonneau et al., 2012). In addition, an identified LLA23-regulated gene encodes branched-chain amino acid transferase (At1g50110), which is coincided with the finding that ZmASR1 influences branched-chain amino acid biosynthesis and maintains kernel yield in maize under water-limited conditions (Virlouvet et al., 2011). Recently, Arenhart et al. (2012) have used a proteomic approach to compare nontransformed and ASR-RNAi rice plants and identified a total of 41 proteins with contrasting expression patterns, most of which are involved in photosynthesis, carbohydrate metabolism and response to stress. However, none of them affected by rice ASR are transcription factors and signaling-related molecules.

The second group of LLA23-regulated genes includes seven transcription factors and five protein kinase genes. These transcription factors may function in the regulation of some stress-inducible genes. For instance, GRAS family transcription factor (At5g41920) is a recently discovered family of plant-specific proteins named after GAI, RGA and SCR, the first three of its members isolated. The GRAS proteins may exert important roles in very diverse processes such as signal transduction, meristem maintenance and development upon various stresses (Ma et al., 2010; Yang et al., 2012). The gene encoding RGA1-like protein (At3g03450) is a DELLA protein which undergoes degradation in response to gibberellin via the 26S proteasome (Piskurewicz et al., 2008). In addition, the protein may be involved in reducing ROS accumulation in response to stress (Ishibashi et al., 2012). The gene encoding pseudoresponse regulator 3 (At5g60100) may affect the period of the circadian clock. Two genes encoding patatin-related phospholipase A (At4g37070) and lysophospholipase (At4g37070) are involved in phospholipid metabolism. Protein phosphatase 2C (At1g16220) combined with SNF1-related protein kinase forms the primary basis of an early ABA signaling module (Hubbard et al., 2010) and WD-repeat protein (At4g33260) may activate respective downstream signal cascades to modulate anthocyanin accumulation and trichome

initiation (Qi et al., 2011). These regulatory proteins are thought to function in further regulating various functional genes under glucose stress condition. SAUR-like auxin-responsive (At4g31320) and jasmonate-responsive (At2g25980) protein genes were identified (Table 2.2), suggesting a link between auxin, jasmonate and sugar-signaling pathways in mediating stress responses. In addition, oxysterol-binding protein-like protein (At5g57240) is related to secondary metabolism and involved in the steroid metabolic processes. Actin depolymerizing factor (At1g01750) is reportedly one of the target proteins in rice seedlings induced by drought stress (Ali and Komatsu, 2006). At present, the functions of most of these protein genes are not fully understood. In addition, substantial amounts of LLA23-induced genes are unknown/hypothetical/putative/expressed proteins, with unknown functions (Table 2.2). In all, the two groups of LLA23-inducible genes correlate with the proposed dual function of the lily ASR protein.

4.2.2. Promoter Analysis of LLA23-Inducible Genes

Sixteen LLA23-inducible genes encoding transcription factors and signaling molecules (Table 2.2) and 25 genes induced by ASR, glucose and drought treatments (Fig. 2.5) were selected for the promoter sequence analysis. Of the 25 genes induced by the three treatments, three (At5g60100, At2g23030 and At2g28960) are transcription factors and signaling genes. Therefore, a total number of 38 genes were examined. Table 2.3 summarizes ABRE, DRE/CBF, sugar-related, CE1, and hormone-responsive core sequences observed in the 38 LLA23-inducible genes identified by the microarray analysis. Among these, 15 genes (39%) contain DRE/CBF-related (G/A)(T/C)CGAC motif in their promoters (Shinozaki and Yamaguchi-Shinozaki, 2007; Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2005), suggesting that these genes are regulated by the DREB1/CBF or DREB2 transcription factors. A grape ASR (VvMSA) protein has reportedly formed heterodimers with drought response element binding (DREB) proteins (Saumonneau et al., 2008). Eleven genes (29%) contain CE1 element (ACGTG) in their promoters, suggesting that these genes are regulated by ASR because the tomato ASR1 was reportedly bound CE1 of ABI4 promoter (Shkolnik and Bar-Zvi, 2008). Also, 25 genes (66%) contain ABRE core element (ACGTG) in their promoters, suggesting that they are ABA-inducible genes similar to *Asr*. Cakir et al. (2003) have demonstrated a specific binding of VvMSA to SURE1 and sucrose box 3 elements in the grape putative monosaccharide transporter promoter. With the exception of five LLA23-inducible genes (At5g53210, At4g33260, At1g22990,

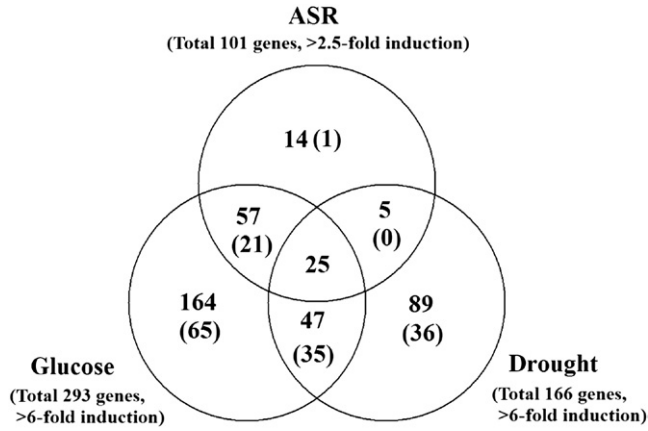

Figure 2.5 Venn diagrams showing the ASR (LLA23)-, glucose-, and drought-upregulated genes identified based on microarray analyses. In total, 101 ASR-, 293 glucose-, and 166 drought-inducible genes were identified via the microarray analysis. The genes identified were grouped into the following seven groups: highly ASR-inducible genes, highly glucose-inducible genes, highly drought-inducible genes, ASR- and glucose-inducible genes, ASR- and drought-inducible genes, glucose- and drought-inducible genes, and ASR-, glucose-, and drought-inducible genes. The number of genes with expression ratios greater than two and halffold induction of expression in *35S::LLA23* plants or sixfold induction of expression in the glucose and drought treatments is indicated. Numbers in parentheses represent the number of genes with a more than two and halffold change in expression ratio in *35S::LLA23* plants or a sixfold change in expression ratio in glucose and drought-stress treatments and a less than twofold change in expression ratio in other stress treatments.

At1g61820, and At5g02860), all the other genes contain at least one of the four sugar-related elements (Sucrose box 3, SURE1, SRE and W-box) in their promoters. This also reflects the existence of novel sugar-responsive and/or other *cis*-acting elements involved in LLA23-inducible gene expression. All the LLA23-inducible genes contain ABA-, auxin-, gibberellin-, ethylene-, and/or salicylic acid-responsive element in their promoters strongly suggesting a complicate network between hormone and ASR-induced signaling pathways.

4.3. Response to Various Stresses Involving ABA

WDS and other abiotic stresses induce various biochemical, physiologic, and adaptive stress in plants. Many of the changes in gene expression that occurs in response to various stresses require the action of ABA (Cutler et al., 2010; Finkelstein and Gibson, 2002; Hubbard et al., 2010; Yamaguchi-Shinozaki and Shinozaki, 2005). Thus, many stress-induced genes are ABA-inducible.

Table 2.3 Promoter sequence analysis in the LLA23-inducible transcription factors and signaling genes identified by the cDNA microarray analysis

Gene	ABRE	DRE/CBF RE*	Sucrose box 3	SURE1	SRE	W-box	CE1	AuxRE	GARE	ERE	SARE
At1g68880	–	−334 to −329	–	–	−699 to −694	−1205 to −1201 −626 to −622 −219 to −215	–	–	–	–	−1206 to −1202 −627 to −623
At3g11020	−786 to −782	−561 to −556	–	–	–	−999 to −995 −104 to −100	–	−1467 to −1462	–	–	−1000 to −996
At5g41920	–	−1036 to −1031 −799 to −794	−451 to −446	–	–	–	−949 to −945 −800 to −796	−388 to −383	–	–	−846 to −842
At1g74500	−1492 to −1488 −1374 to −1370 −903 to −899	–	−1313 to −1308	–	–	–	−69 to −65	–	−318 to −312	−470 to −463	−1323 to −1319
At3g03450	−1214 to −1210	–	−789 to −784 −170 to −165	–	−881 to −876 −701 to −696 −387 to −382	−1482 to −1478	–	−974 to −969	−916 to −910	–	–

Continued

Table 2.3 Promoter sequence analysis in the LLA23-inducible transcription factors and signaling genes identified by the cDNA microarray analysis—cont'd

Gene	RE* ABRE	DRE/CBF	Sucrose box 3	SURE1	SRE	W-box	CE1	AuxRE	GARE	ERE	SARE
At5g60100	–	−1486 to −1481 −1081 to −1076	−739 to −734	–	−274 to −269	–	–	–	–	–	−982 to −978 −33 to −29
At5g53210	–	–	–	–	–	–	–	−692 to −687	–	−1369 to −1362 −652 to −645	–
At4g37070	−868 to −864 −592 to −588	−1075 to −1070 −245 to −240	–	–	−877 to −872	−704 to −700	–	−285 to −280	–	−1161 to −1154 −312 to −305	−445 to −441 −74 to −70
At3g15650	–	–	−1412 to −1407 −1333 to −1328	−1220 to −1212	–	–	–	−282 to −277	–	−74 to −67	–
At1g16220	−513 to −509 −417 to −413	−107 to −102	−476 to −471	–	–	−1081 to −1077 −357 to −353	−1352 to −1348	−268 to −263 −220 to −215	–	–	−1082 to −1078 −358 to −354

At2g23030	−246 to −242	–	−894 to −889 −847 to −842 −753 to −748 −235 to −230	−904 to −896	–	−1124 to −1120 −151 to −147	–	−544 to −539	–	−882 to −875	−1033 to −1029
At4g33260	−1384 to −1380	−321 to −316	–	–	–	–	–	−439 to −434	−921 to −915	–	–
At4g36180	–	−1444 to −1439	−740 to −735	–	–	–	−398 to −394	–	−870 to −864	–	–
At1g54510	–	−1363 to −1358	–	–	–	−100 to −96	–	−717 to −712	−18 to −12	–	−101 to −97
At1g51940	–	–	−1255 to −1250 −133 to −128 −91 to −86	–	–	−1458 to −1454 −1351 to −1347 −386 to −382	–	–	−1474 to −1468	–	−1459 to −1455 −1352 to −1348 −1015 to −1011 −490 to −486 −387 to −383 −306 to −302

Continued

Table 2.3 Promoter sequence analysis in the LLA23-inducible transcription factors and signaling genes identified by the cDNA microarray analysis—cont'd

Gene	RE* ABRE	DRE/CBF	Sucrose box 3	SURE1	SRE	W-box	CE1	AuxRE	GARE	ERE	SARE
At2g28960	–	–	−73 to −68	–	–	–	–	–	−1208 to −1202 −648 to −642 −635 to −629	–	−1374 to −1370 −1261 to −1257 −607 to −603 −315 to −311
At3g01190	−433 to −429	−315 to −310	–	–	−968 to −963	−1026 to −1022	–	–	−1256 to −1250	–	−1338 to −1334 −1027 to −1023 −735 to −731
At1g54890	−158 to −154	–	−384 to −379	–	–	−1152 to −1148 −420 to −416 −202 to −198	–	–	−1129 to −1123 −890 to −884	–	−1153 to −1149 −823 to −819 −520 to −516 −421 to −417 −203 to −199

At4g30660	−1423 to −1419 −993 to −989 −836 to −832 −117 to −113	−1148 to −1143	–	–	–	−1209 to −1205 −924 to −920 −143 to −139	−373 to −369	−1001 to −996	–	–	−1210 to −1206 −144 to −140
At1g22990	−1087 to −1083	−553 to −548	–	–	–	–	−1462 to −1458	–	–	−465 to −458	−1104 to −1100
At4g21680	–	–	−1115 to −1110 −1063 to −1058	–	−1132 to −1127	−247 to −243	−358 to −354 −102 to −98	–	−1465 to −1459 −383 to −377	–	−1388 to −1384
At4g28040	−60 to −56	–	−1341 to −1336 −1088 to −1083 −774 to −769 −608 to −603	−617 to −609	−1156 to −1151	−1016 to −1012	–	–	–	–	−138 to −134
At2g37890	−1250 to −1246	−533 to −528	−472 to −467	–	–	−814 to −810	–	−107 to −102	−286 to −280	–	−1358 to −1354 −1284 to −1280

Continued

Table 2.3 Promoter sequence analysis in the LLA23-inducible transcription factors and signaling genes identified by the cDNA microarray analysis—cont'd

Gene	RE* ABRE	DRE/CBF	Sucrose box 3	SURE1	SRE	W-box	CE1	AuxRE	GARE	ERE	SARE
At3g60140	–	–	−1093 to −1088 −990 to −985 −621 to −616	–	–	−350 to −346	–	–	–	–	−506 to −502
At3g24840	−1010 to −1006 −967 to −963 −920 to −916	–	−1426 to −1421 −213 to −208	–	–	−1203 to −1199 −691 to −687 −630 to −626 −387 to −383	−763 to −759	−137 to −132	−1191 to −1185	–	−804 to −800 −692 to −688 −631 to −627 −388 to −384
At1g15210	−996 to −992	–	–	–	–	−1281 to −1277 −391 to −387	–	–	−795 to −789	–	−934 to −930 −731 to −727 −510 to −506 −392 to −388
At1g61820	−639 to −635	–	–	–	–	–	–	–	–	–	−134 to −130

At4g13660	–	–	–	–	−197 to −192	−1476 to −1472 −747 to −743 −346 to −342 −292 to −288	−445 to −441	−126 to −121	–	−699 to −692	−799 to −795 −748 to −744 −719 to −715 −347 to −343 −293 to −289
At5g64860	−125 to −121 −114 to −110	–	–	–	−6 to −1	−320 to −316	−1103 to −1099 −131 to −127	–	–	–	−394 to −390 −321 to −317
At5g57240	−756 to −752	–	–	–	–	−761 to −757	–	–	−750 to −744	–	−952 to −948 −86 to −82
At3g13674	–	–	−1490 to −1485	–	−1275 to −1270	−596 to −592	−153 to −149	–	–	–	−597 to −593
At3g19390	−1500 to −1496 −203 to −199	–	−782 to −777	–	–	−1325 to −1321	–	–	–	–	−1326 to −1322

Continued

Table 2.3 Promoter sequence analysis in the LLA23-inducible transcription factors and signaling genes identified by the cDNA microarray analysis—cont'd

Gene	RE* ABRE	DRE/CBF	Sucrose box 3	SURE1	SRE	W-box	CE1	AuxRE	GARE	ERE	SARE
At5g02860	−1353 to −1349 −165 to −161	–	–	–	–	–	–	–	−1374 to −1368	−409 to −402	−1333 to −1329 −733 to −729 −624 to −620 −357 to −353 −220 to −216
At5g19340	−993 to −989 −167 to −163	–	–	–	−961 to −956 −901 to −896 −304 to −299	−706 to −702	–	−1343 to −1338 −1119 to −1114 −180 to −175	–	–	–
At3g15140	−384 to −380	−10 to −5	–	–	–	−1425 to −1421 −947 to −943	–	−840 to −835	–	–	–

At3g12700	−824 to −820 −671 to −667	−1231 to −1226	−857 to −852	–	–	−1462 to −1458 −1456 to −1452	–	–	−39 to −33	−922 to −915 −678 to −671 −79 to −72	−1457 to −1453 −937 to −933
At2g20480	−98 to −94 −81 to −77	–	–	–	–	−934 to −930	–	–	−663 to −657 −590 to −584 −340 to −334	–	−1446 to −1442 −1374 to −1370
At3g22570	−309 to −305	–	–	–	−924 to −919 −728 to −723	–	–	–	−50 to −44	–	−264 to −260

*Regulatory elements (RE): ABRE, ACGTG; DRE/CBF, (G/A)(T/C)CGAC; Sucrose box 3, AAATCA; SURE1, AATAGAAAA; SRE, TTATCC; W-box, TGACT; CE1, CACCG; AuxRE, TGTCTC; GARE, TAACAA(G/A); ERE, A(A/T)TTCAAA; SARE, TTGAC.

Most genes that respond to environmental stimuli such as drought, cold, and salt stresses are also induced by exogenous ABA treatment (Cai et al., 2011). In vegetative tissues, a rapid increase in ABA levels promotes stomatal closure and prevents stomatal opening, thereby reducing transpirational water loss under water stress conditions. During late embryogenesis, ABA promotes the acquisition of desiccation tolerance and seed dormancy, and inhibits seed germination (Fujita et al., 2011; Koornneef et al., 2002; Seiler et al., 2011). In addition, ABA can hasten the initiation of fruit ripening (Chen et al., 2011).

A sizable amount of literature has been published on the characterization of ABA signal transduction cascades (Cutler et al., 2010; Fujita et al., 2011; Hubbard et al., 2010; Liu, 2012). ABA-responsive gene expression is regulated by transcription factors, receptors, protein kinase, phosphatase, chromatin-remodeling factors, and secondary messengers, such as Ca^{2+}, cADPR, and inositol triphosphate, which have been implicated in ABA-mediated responses (Lemtiri-Chlieh et al., 2003; Roelfsema and Hedrich, 2010;Viswanathan and Zhu, 2002). Moreover, ABA signaling is regulated by mRNA maturation and stability, microRNA levels, nuclear specking, and protein degradation (Nonogaki, 2010; Xie et al., 2005). Thus, ABA signaling is clearly involved in a complex network of both positively and negatively regulating components.

Although the expression of the *DS2* gene in potato (*Solanum tuberosum*) is ABA-independent (Dóczi et al., 2005), *Asr* genes are typically upregulated by ABA upon dehydration (Huang et al., 2000; Philippe et al., 2010; Silhavy et al., 1995), salt (Dai et al., 2011; Kalifa et al., 2004b; Yang et al., 2005), and cold treatments (Hsu et al., 2011; Kim et al., 2009; Schneider et al., 1997). *Asr* genes also respond to pathogen attacks (Liu et al., 2010; Muñiz et al., 2012). In addition, *Asr* genes have been suggested to play important roles in plant developmental processes, such as senescence (Jeanneau et al., 2002), pollen maturation (Wang et al., 1998), flowering (Dóczi et al., 2005; Gilad et al., 1997), flower (Dóczi et al., 2005; Gilad et al., 1997), fruit development (Cakir et al., 2003; Canel et al., 1995; Chen et al., 2011), and glucose metabolism (Cakir et al., 2003; Frankel et al., 2007; Saumonneau et al., 2012; Shkolnik and Bar-Zvi, 2008). Recently, Arenhart et al. (2012) indicated that the *ASR5* gene is differentially regulated in the aluminum-tolerant rice and the obtained ASR-RNAi plants exhibit aluminum susceptibility. The ectopic overexpression of *LLA23* and *MpAsr* in transgenic *Arabidopsis* is insensitive to ABA and increases plant tolerance to drought and salt stresses (Dai et al., 2011; Yang et al., 2005). In addition, ectopic

SlASR1 overexpression increases salt tolerance in tobacco and influences glucose metabolism in potato (Frankel et al., 2007; Kalifa et al., 2004b). The overexpression of tomato ASR1 in *Arabidopsis* produces an *abscisic acid-insensitive 4 (abi4)* phenotype, wherein seed germination is not sensitive to inhibition by ABA, glucose, and NaCl (Shkolnik and Bar-Zvi, 2008).

4.4. Response to Glucose Stress

Glucose, one of the hydrolytic products of sucrose, is a key metabolic signal that controls the expression of many genes via diverse mechanisms, such as transcription, translation, and modification of mRNA and protein stability (Li et al., 2006; Matiolli et al., 2011; Price et al., 2004; Rolland et al., 2006). Despite extensive studies on the involvement of ABA in glucose sensing and signaling, information regarding the interactions between glucose and ABA signaling pathways and their integration with developmental programs is still far less complete. Thus far, only a number of regulators, such as MYBs, SUSIBA2, and MBF1c, which are required for a sugar have been identified (Abe et al., 2003; Lu et al., 2002; Matiolli et al., 2011; Sun et al., 2005). Additional regulators and signaling components are expected to be found to unravel the regulatory networks that transmit sugar signals. ASR, a newly identified regulator, is reportedly involved in sugar and ABA signaling (Cakir et al., 2003; Saumonneau et al., 2012). Considering ABA/sugar interactions have been reported for a multitude of processes, a thorough understanding of the underlying molecular mechanisms of sugar and ABA interactions is important.

Wild-type *Arabidopsis* (Col) plants under 3% glucose stress for 12 h or under drought stress conditions were analyzed using a microarray in parallel with unstressed wild-type *Arabidopsis* (Col) plants. Only the genes with high significance ($P < 0.05$) and at least twofold changes in expression were selected. Thus, 1126 and 819 genes with expression ratios (3% glucose/unstressed; drought/unstressed) greater than twofold induction were identified as glucose- and drought-inducible, respectively. Similarly, 757 and 709 genes with expression ratios greater than twofold repression were identified as glucose- and drought-repressive, respectively. Our analysis is consistent with other microarray analysis showing that glucose treatment regulates a broad range of gene functions (Price et al., 2004; Thimm et al., 2004). In this study, 293 glucose-upregulated, 166 drought-upregulated, 144 glucose-downregulated, and 116 drought-downregulated genes with induction or repression greater than sixfold were used for comparison.

4.4.1. Gene Identification and Cross Talk among LLA23-, Glucose-, and Drought-Inducible Responses

The Venn diagram in Fig. 2.5 shows that 101, 293, and 166 genes were identified as LLA23-, glucose-, and drought-inducible genes with at least two and halffold induction of expression in LLA23-overexpressing plants or at least sixfold induction of expression in the glucose and the drought treatment. Although drought is considered to be the most severe stress that affects plant growth, the number of glucose-inducible genes is more pronounced than that of drought-inducible genes, which suggests that aside from osmotic stress, glucose acts as a metabolic signal that regulates the expression of many genes via diverse mechanisms in the cell. Of the three treatments, the least effective one is the group of genes induced by LLA23, which indicates that genes affected by ASR represent only a small fraction of the stress-inducible genes. These genes include substantial amounts of reported drought and osmotic stress-inducible genes, which indicates that the microarray system for finding these genes is functioning properly.

The Venn diagram analysis reveals difference and cross talk in gene expression among the drought-, glucose-, and ASR-induced responses (Fig. 2.5). Up to 82 genes were identified as glucose- and LLA23-inducible genes, whereas only 30 genes were induced by both drought and LLA23-overexpressing treatments, and 72 genes were induced by both glucose and drought. Li et al. (2006) have reported that genes induced by both glucose and ABA are involved in stress responses, indicating overlapping regulation by glucose and ABA. Moreover, 25 genes were induced under all three stresses, as indicated by the cross in Table 2.2. These genes encoding functional proteins such as detoxification, LEA, transporter, cell wall- and defense-related proteins that probably function in stress tolerance and others are involved in carbohydrate metabolism, secondary metabolism, amino acid and protein metabolism, transcript regulation, and signal transduction. These results indicate a strong association in the expression of stress-responsive genes among the three signaling transduction pathways. Despite the dense overlaps among glucose-, drought-, and LLA23-inducible genes, we found a substantial amount of stress-inducible genes that were highly induced by each treatment. These highly inducible genes are discussed in the following section.

4.4.2. Highly Stress-Inducible Genes

Among the stress-inducible genes identified, substantial amounts of genes are highly induced by each stress. In this study, genes with expression ratios

greater than threefold in the LLA23-overexpressing treatment or greater than sixfold in the glucose and drought treatments, and less than twofold in the other two induced treatments were classified as highly stress-inducible genes. Thus, only one LLA23-, 65 glucose-, and 36 drought-inducible genes induced highly in each stress treatment were identified (Table 2.4). As shown in Fig. 2.6, real-time quantitative polymerase chain reaction (Q-PCR) analysis of the hypothetical protein (At4g27510), unknown (At3g17990), and early-responsive to dehydration 7 (ERD7, At2g17840) confirmed that they are highly inducible LLA23-, glucose-, and drought-inducible genes, respectively. The expression profiles obtained using Q-PCR analysis are consistent with those obtained through microarray analysis. Among the genes highly induced by 3% glucose, most are categorized as cell wall-related, stress- and defense-related, involved in amino acid and protein metabolism, and as functional proteins responsive to osmotic stress, such as detoxification LEA, transporter, and water channel proteins. Among the genes highly induced by drought stress, a lot of them are functional and stress-related proteins (Table 2.4). In addition, we could find a close-to-constitutively expressed gene (At1g11890), whose expression ratio is 1.00 in the glucose treatment, 0.99 in the LLA23-overexpressing treatment, and 1.01 in the drought treatment. This gene may be useful as an internal control in the microarray analysis.

4.4.3. LLA23-, Glucose-, and Drought-Repressive Genes

Analysis of stress-repressive genes is also important in understanding molecular responses to abiotic stresses. As previously described, 204 genes were identified as LLA23-repressive, 757 were identified as glucose-repressive, and 709 genes were identified as drought-repressive. However, only the genes with a more than two and halffold change in expression ratio in the LLA23-overexpressing treatment or a more than sixfold change in expression ratio in the glucose and drought treatments were classified based on their expression profiles (Fig. 2.7). A total of 12 LLA23-, 144 glucose stress-, and 116 drought stress-downregulated genes were identified and classified using microarray analysis (Tables 2.2 and 2.5).

Among these repressive genes, fewer were downregulated by LLA23. Six genes encoding one heat shock protein 101(At1g74310), one mitochondrial dicarboxylate carrier (At4g24570), one 5-adenylylsulfate reductase (At4g04610), and three hypothetical/unknown proteins were repressed only by LLA23 (Table 2.2). Of these repressive genes, 5-adenylylsulfate reductase is photosynthesis-related. In addition, one gene encodes PRH26 protein (At4g21990) located in chloroplast was repressed by both LLA23 and

Table 2.4 Number of highly glucose- and drought-inducible genes* involved in different functional groups in *Arabidopsis*

Functional category	Gene number	Representative gene names
Highly inducible genes by glucose only (65)		
Transcription factor	1	Shatterproof 1 (At3g58780)
Signaling	3	Calcium sensor homolog (At5g24270), WD-40 repeat protein MSI2 (At2g16780), serine-rich protein (At5g37010)
Detoxification	3	Peroxidase ATP3a (At5g64100), peroxidase ATP12a (At1g05250), peroxidase ATP13a (At4g26010)
LEA protein	1	Late embryogenesis abundant protein-related (At5g54370)
Transporter	1	Lysine and histidine-specific transporter (At1g47670)
Water channel protein	1	Like (aquaporin) mipC protein (At5g60660)
Cell wall-related	9	Beta-fructofuranosidase 1 (At3g13790), endo-1,4-beta-glucanase (At1g65610), glycine-rich protein (At4g29020), N-hydroxycinnamoyl/benzoyltransferase (At5g41040), nucleoside triphosphate hydrolases (At3g03060), pectin lyase-like superfamily protein (At3g61490), SGNH hydrolase-type esterase superfamily protein (At1g75880), similar to extensin-like protein (At4g02270), UTP-glucose glucosyltransferase (At5g66690)
Defense-related	4	Germin precursor oxalate oxidase (At4g14630), nudix hydrolase homolog 13 (At3g26690), CAP (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1) (At1g50060), probable wound-induced protein (At4g10270)
Carbohydrate metabolism	3	Glutamate dehydrogenase 2 (At5g07440), glyceraldehyde-3-phosphate dehydrogenase (At1g79530), similarity to alpha2,8-sialyltransferase (At1g08280)
Fatty acid metabolism	3	Two GDSL-like lipase/acylhydrolase (At1g54020, At4g30140), stearoyl acyl carrier protein desaturase (At1g43800)
Secondary metabolism	1	Terpene synthase (At3g29410)
Cellular metabolism	2	Endomembrane protein 70 (At2g24170), transketolase precursor (At2g45290)

Amino acid and protein metabolism	9	60S ribosomal protein L27 (At2g32220), cysteine protease XCP1 (At4g35350), E2 ubiquitin-conjugating enzyme (At1g50490), ubiquitin extension protein (At1g23410), homocysteine S-methyltransferase AtHMT-1 (At3g25900), two S-adenosyl-L-methionine-dependent methyltransferases (At5g37990, At5g10830), serine carboxypeptidase isolog (At1g11080), subtilisin-like serine protease (At4g20430)
Homeostasis	1	Peroxiredoxin (At1g60740)
Cytoskeleton	1	Putative kinesin heavy chain (At2g22610)
Jasmonate-related	1	Jasmonate-inducible protein (At1g52050)
DNA, nucleus	1	Replication factor A (At5g08020)
Senescence-related	1	Rhodanese/cell cycle control phosphatase (At2g21045)
Unclassified	19	Three expressed proteins (At3g02885, At2g20515, At5g62165), seven hypothetical proteins (At3g20260, At1g34355, At1g20590, At1g77270, At3g24000, At4g01780, At2g44190), four putative predicted proteins (At4g29520, At5g15120, At5g14920, At5g40730), five unknown proteins (At5g45700, At5g53250, At3g17990, At2g01610, At2g20870)
Highly inducible genes by drought only (36)		
Signaling	2	Two phosphatase-2C (At5g27930, At3g05640)
Drought-related	1	Early-responsive to dehydration 7 (At2g17840)
Transporter	3	Peptide transporter (At1g22570), tetracycline transporter protein (At2g16990), major facilitator superfamily protein (At1g67300)
Cell wall-related	1	UDP glucose 4-epimerase (At4g23920)
Defense-related	2	Aldehyde dehydrogenase homolog (At1g54100), mannose-binding lectin superfamily protein (At1g52000)
Carbohydrate metabolism	1	Alpha-hydroxynitrile lyase HNL4 (At5g10300)
Fatty acid metabolism	1	Peroxisomal citrate synthase (At2g42790)

Continued

Table 2.4 Number of highly glucose- and drought-inducible genes* involved in different functional groups in *Arabidopsis*—cont'd

Functional category	Gene number	Representative gene names
Cellular metabolism	4	Branched-chain alpha keto-acid dehydrogenase (At1g21400), p53 regulated PA26-T3 nuclear protein (At3g55910), polyamine oxidase 2 (PAO2) (At2g43020), similar to glucose 1-dehydrogenase (At1g62610)
Amino acid and protein metabolism	3	Aspartyl protease family protein (At5g37540), beta-VPE (vacuolar processing enzyme) (At1g62710), branched-chain amino acid aminotransferase (At1g10070)
Cytoskeleton	1	Microtubule-associated protein (At2g05630)
DNA, nucleus	1	Zinc finger protein (At1g78600)
Dormancy	1	Dormancy-associated protein (At1g28330)
Unclassified	15	Expressed protein (At4g14270), five hypothetical proteins (At1g80160, At1g68500, At1g20440, At1g55760, At1g11170), putative protein (At4g18530), eight unknown proteins (At2g41190, At1g69260, At2g15890, At1g07040, At5g47550, At5g50450, At3g03310, At2g15960)
Highly inducible genes by both glucose and drought (35)		
Transcription factor	2	Abscisic acid responsive elements-binding factor (At1g49720), pseudo-response regulator 1 (At5g61380)
Signaling	2	Phosphatase 2C (At2g25620), pyruvate water dikinase (At5g26570)
Stress-related	5	Two alcohol dehydrogenase (At1g77120, At3g29250), low-temperature and salt-responsive protein (At4g30650), drought-induced protein like (At4g15910), DnaJ protein (At1g56300)
Transporter and ion-binding	4	$Ca2^{+}$-transporting ATPase-like protein (At5g57110), membrane channel protein (At2g28900), chloroplast protein (At4g03320), XB3 ortholog 1 (At2g28840)
Cell wall-related	1	Beta-galactosidase (At5g63810)
Defense-related	1	Putative major latex protein (At4g23680)

Carbohydrate metabolism	1	Isoamylase-like protein (At4g09020)
Fatty acid metabolism	1	GDSL-like lipase/acylhydrolase superfamily protein (At4g01130)
Secondary metabolism	1	Nitrile-specifier protein NSP3 (At3g16390)
Cellular metabolism	3	GTP cyclohydrolase (At2g22450), aminotransferase (At3g08860), TFL1 (terminal flower 1) (At2g27550)
Amino acid and protein metabolism	1	Methionine aminopeptidase I (MAP1) (At1g13270)
Permeability	1	Cyclophilin (CYP2) (At2g21130)
Unclassified	12	Expressed protein (At2g38465), two hypothetical proteins (At3g46640, At1g17830), six putative predicted proteins (At3g47860, At4g34950, At4g33980, At5g14550, At5g03870, At5g58600), three unknown proteins (At2g22470, At2g39030, At5g23240)

*Genes with expression ratios greater than sixfold for glucose and/or drought treatment, but less than twofold for the other induced treatments are defined as highly glucose- and/or drought-inducible genes.

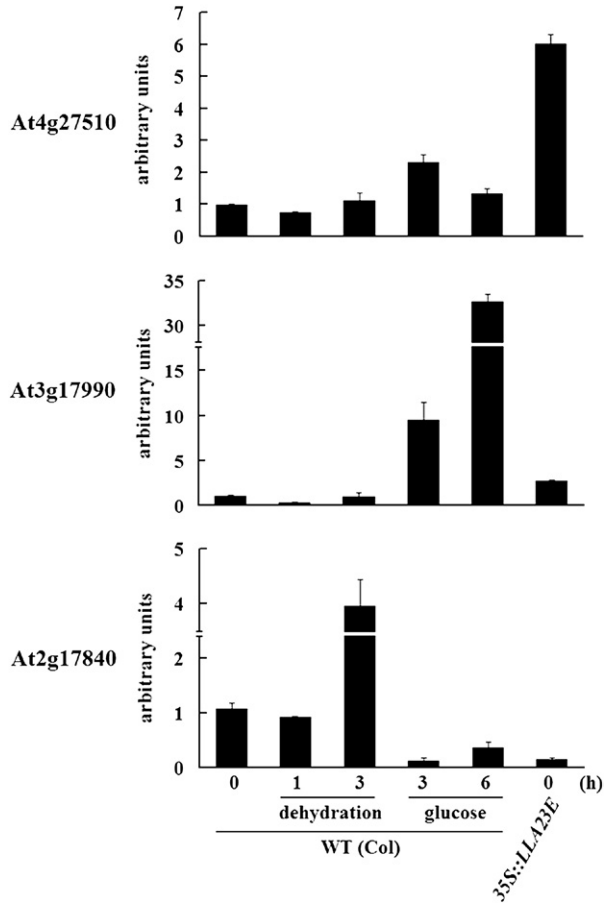

Figure 2.6 Q-PCR analysis of highly inducible ASR (LLA23)-, glucose-, or drought-inducible genes. Results for a highly LLA23-inducible gene (At4g27510); a highly glucose-inducible gene (At3g17990); and a highly drought-inducible gene (At2g17840). Wild-type (Col) and transgenic *35S::LLA23E* plants were grown on soil for 2 weeks. For dehydration, wild-type plants were removed and dried on the air for 1 and 3 h. For glucose stress, wild-type plants were grown in the MS medium for 2 weeks. The plants were removed and shaked in the MS solution containing 3% glucose for 3 and 6 h. Total RNA was isolated from wild-type and transgenic plants and the level of mRNA was determined by Q-PCR. The data of Q-PCR analysis were obtained from three independent experiments. Error bars represent SD.

drought (Table 2.2). We also found photosynthesis-related genes, such as carbonic anhydrase (At3g52720 and At3g01500), PSI type II chlorophyll a/b-binding protein (At1g19150) and dihydrodipicolinate reductase-like protein (At5g52100) repressed by drought and glucose stresses (Table 2.5). Including those genes with expression ratios greater than twofold but less than two

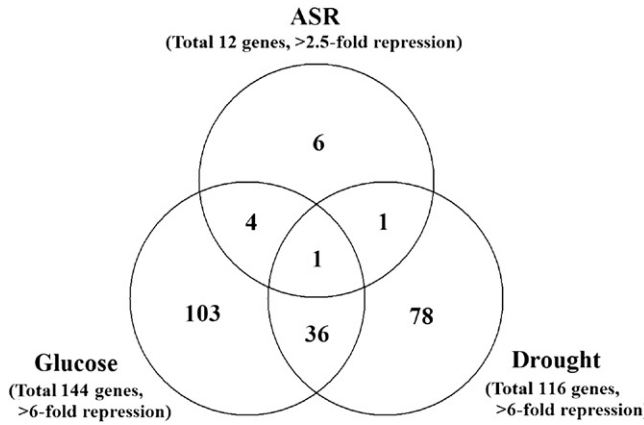

Figure 2.7 Venn diagrams showing the ASR (LLA23)-, glucose-, and drought-downregulated genes identified basis on microarray analyses. In total, 12 ASR-, 144 glucose-, and 116 drought-repressive genes were identified via the microarray analysis. The genes identified were grouped into the following seven groups: highly ASR-repressive, highly glucose-repressive, highly drought-repressive, ASR- and glucose-repressive genes, ASR- and drought-repressive genes, glucose- and drought-repressive genes, and ASR-, glucose-, and drought-repressive genes. The number of genes with a more than two and halffold change in expression ratio in *35S::LLA23* plants or a sixfold change in expression ratio in the glucose and drought treatments is indicated.

and halffold change in expression ratio in the LLA23-overexpressing treatment, 37 genes such as Rubisco activase, Rubisco subunit-binding proteins, plastocyanin, phytocyanin, and components of photosystems I and II, were downregulated. These results are consistent with a previous report that indicated that water stress inhibits photosynthesis (Seki et al., 2002; Tezara et al., 1999). For glucose and/or drought-stress treatment, transcription factors and signaling genes consist of 20% of downregulated genes in stressed plants. Functional proteins, such as detoxification enzymes, transporters, enzymes for osmoprotectant synthesis, cell wall-related, stress- and defense-related proteins, were also significantly repressed. We observed that the expression of some stress-responsive genes in wild-type plants is upregulated, whereas some are downregulated. Although the apparent inconsistencies in stress responsiveness are not easily explained, they are not without precedent (Kang et al., 2002; Seki et al., 2002; Price et al., 2004). Interestingly, auxin-induced protein (At1g29500, At1g29510 and At1g04250), zeaxanthin epoxidase precursor (At5g67030) for ABA biosynthesis, and gibberellin-regulated protein (At1g22690) genes were also identified (Table 2.5), suggesting a link between auxin, ABA, gibberellins, and signaling pathways of sugar or drought stress.

Table 2.5 Number of glucose- and drought-repressive genes* involved in different functional groups in *Arabidopsis*

Functional category	Gene number	Representative gene names
Repressive genes by glucose only (103)		
Transcription factor	12	AP2 domain-containing protein RAP2 (At1g22190), two dof zinc finger proteins (At5g62430, At1g51700), Myc-related bHLH transcription factor (At3g59060), NAC transcription factor (At2g17040), promoter-binding protein (At5g39660), B-box zinc finger protein (At2g21320), transcriptional regulator (At4g00050), zinc finger transcription factor (PEI1) (At3g55980), RING-H2 finger protein RHA3a (At1g49200), sigma-like factor (At5g24120), WRKY transcription factor (At3g56400)
Signaling	13	Calmodulin-like protein (At2g41100), flowering signals mediating protein FT (At1g65480), five protein kinases (At1g66880, At2g23200, At1g69730, At2g40270, At4g02420), four receptor protein kinases (At2g31880, At1g65790, At1g51805, At5g48540), two serine/threonine protein kinases (At4g23180, At5g01820)
Detoxification	1	Glutathione S-transferase TSI-1 (At1g10370)
Osmoprotectant synthesis	1	Trehalose-6-phosphate synthase (At1g70290)
Cold-related	1	Calcium-dependent lipid-binding family protein (At4g34150)
Transporter	6	ABC transporter protein 1-like (At5g64840), ammonium transport protein (AMT1) (At4g13510), cyclic nucleotide-regulated ion channel protein (At2g46440), membrane channel like protein (At4g17340), oligopeptide transporter (At4g10770), similarity to ion channel (At3g17700)
Cell wall-related	1	Cell wall protein ECS1 (At1g31580)
Defense-related	3	Two disease resistance proteins (At1g66090, At3g50950), flax rust resistance protein (At1g72930)
Carbohydrate metabolism	2	Beta-amylase (At4g17090), sucrose-phosphate synthase (At4g10120)
Fatty acid metabolism	1	Microbody NAD-dependent malate dehydrogenase (At5g09660)

Secondary metabolism	3	Anthocyanin 5-aromatic acyltransferase (At2g39980), squalene epoxidase (At4g37760), squalene monooxygenase (At5g24150)
Cellular metabolism	7	FRO2-like protein (At5g49730), member of the peroxin11 (PEX11) gene family (At3g47430), neoxanthin cleavage enzyme-like protein (At4g19170), PDX1-like protein 4 (At2g38210), 1-aminocyclopropane-1-carboxylate oxidase (At1g03400), oxidoreductase (At2g37540), similarity to alternative NADH-dehydrogenase (At1g07180)
Amino acid and protein metabolism	3	E2 ubiquitin-conjugating enzyme (At2g33770), glycine decarboxylase P-protein 1 (At4g33010), alanine aminotransferase (At1g70580)
Protease inhibitor	1	Cysteine proteinase inhibitor B (At2g40880)
DNA, nucleus	1	DNA helicase (At5g35970)
Cytochrome P450	1	Cytochrome P450 (At3g26230)
Auxin-related	1	Auxin-induced protein (At1g29500)
ABA biosynthesis	1	Zeaxanthin epoxidase precursor (At5g67030)
Unclassified	44	Five expressed proteins (At4g14365, At2g25735, At1g73800, At1g69523, At4g02075), 10 hypothetical proteins (At1g70700, At2g24600, At1g21250, At1g65190, At5g02180, At2g46790, At2g32160, At3g50480, At2g41120, At1g23390), six putative proteins (At4g37610, At4g26850, At4g27030, At4g28290, At4g36500, At3g56290), 22 unknown proteins (At1g14870, At5g55970, At2g26530, At3g01060, At1g14200, At1g16720, At2g30510, At1g33110, At1g50020, At5g24210, At5g44580, At1g57990, At2g42750, At5g47610, At1g76110, At1g29700, At5g06980, At3g22240, At5g44060, At1g23840, At1g52870, At3g56360), predicted protein (At2g04039)
Repressive genes by drought only (78)		
Transcription factor	4	Two bZIP transcription factor family proteins (At5g28770, At5g15830), helix–loop–helix DNA-binding protein (At1g73830), RING zinc finger protein (At3g06140)
Signaling	5	Cobalamin biosynthesis protein (At5g13630), NPK1-related protein kinase 2 (At1g12110), two receptor protein kinases (At4g04540, At4g34220), photomorphogenesis repressor protein (At5g23730)

Continued

Table 2.5 Number of glucose- and drought-repressive genes* involved in different functional groups in *Arabidopsis*—cont'd

Functional category	Gene number	Representative gene names
Detoxification	1	Glutathione transferase-like protein (At3g43800)
Transporter	4	Nodulin MtN21/EamA-like transporter protein (At2g37460), oligopeptide transporter (At4g16370), peptide transporter (At1g52190), phosphate transporter (At3g26570)
Water channel protein	1	Aquaporin (At2g36830)
Cell wall-related	13	Anthranilate N-hydroxycinnamoyl/benzoyltransferase (At3g50270), two beta-1,3-glucanase-like proteins (At5g42720, At4g18340), beta-glucosidase (At1g26560), cinnamyl alcohol dehydrogenase (At3g19450), endo-xyloglucan transferase (At4g37800), expansin (At-EXP1) (At1g69530), invertase (At1g22650), pectinesterase (At3g10720), pectate lyase (At4g24780), retinal glutamic acid-rich protein (At5g28630), storage protein (At4g24350), similarity to extensin-like protein (At1g49750)
Defense-related	1	Lectin (At3g16530)
Fatty acid metabolism	3	Lipase-like protein (At3g48460), omega-3 fatty acid desaturase (At3g11170), delta 9 desaturase (At3g15850)
Secondary metabolism	1	Similarity to chalcone-flavonone isomerase (At5g05270)
Cellular metabolism	3	5-adenylylphosphosulfate reductase (At1g62180), phosphoethanolamine N-methyltransferase (At1g73600), S-adenosylmethionine decarboxylase (At5g15950)
Amino acid and protein metabolism	5	ACT domain repeat 3 (At1g76990), aminomethyltransferase-like precursor (At1g11860), glycine cleavage system H protein precursor (At1g32470), L-aspartate oxidase-like protein (At5g14760), RING-H2 finger protein (At4g30400)
Homeostasis	2	Cadmium-transporting ATPase-like protein (At4g30110), H^+-ATPase 11 (At5g62670)
Photosynthesis	3	Carbonic anhydrase (CAH1) (At3g52720), dihydrodipicolinate reductase-like protein (At5g52100), PSI type II chlorophyll a/b-binding protein (At1g19150)
Cytoskeleton	1	Nitrilase-associated protein (At5g15600)
DNA, nucleus	2	RNA-binding protein (At1g09340), zinc finger protein 3 (At5g16540)
Auxin-related	1	Auxin-induced protein (At1g04250)
Gibberellin-related	1	Gibberellin-regulated protein (At1g22690)

Unclassified	27	Four expressed proteins (At1g55910, At3g17610, At4g16985, At1g14345), seven hypothetical proteins (At4g15430, At1g21050, At1g19380, At2g30010, At3g03770, At2g34620, At3g59400), five predicted proteins (At4g28080, At5g19970, At5g61130, At4g36540, At4g39770), 11 unknown proteins (At3g23550, At5g24660, At5g54120, At1g15290, At5g54130, At1g75750, At1g19670, At1g44000, At1g67480, At2g35860, At2g44210)
Repressive genes by both glucose and drought (36)		
Transcription factor	7	B-box zinc finger protein (At2g31380), CONSTANS-like 1 (At5g15850), two Myb-related transcription factors (At2g46830, At3g09600), two DNA-binding proteins (At1g01060, At1g68840), flowering-time gene (At3g02380)
Signaling	2	Two serine–threonine protein kinases (At4g23290, At1g78290)
Transporter and ion-binding	4	Amino acid permease 6 (At5g49630), copper transport protein (At3g46900), membrane related protein CP5 (At1g55960), calcium binding protein (At2g41090)
Cell wall-related	2	Expansin (At2g40610), cell wall-associated kinase (At1g16260)
Defense-related	1	Hairpin-induced gene (HIN1) (At2g35960)
Carbohydrate metabolism	2	Beta-amylase-like protein (At5g18670), starch synthase (At1g32900)
Secondary metabolism	2	Beta-carotene hydroxylase (At5g52570), steroid dehydrogenase homolog (At3g50560)
Cellular metabolism	1	Hydrolase-like protein (At4g12830)
Homeostasis	1	Glutaredoxin (At1g06830)
Photosynthesis	1	Carbonic anhydrase (At3g01500)
Auxin-related	1	Auxin-induced protein (At1g29510)
Unclassified	12	Four hypothetical proteins (At2g41250, At3g22060, At4g01080, At4g12980), two putative proteins (At5g19240, At5g59080), six unknown proteins (At3g17330, At3g26960, At1g04530, At5g22390, At2g30520, At3g12320)

*Genes with expression ratios greater than sixfold for glucose and/or drought treatment.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The lily ASR is induced through desiccation-associated ABA signaling transduction in the pollen. We propose a dual role for LLA23, as a regulator and a protective molecule, upon exposure to water deficits. A genome-wide microarray and promoter analysis suggests a strong association in the expression of stress-responsive genes among ASR-overexpressing, sugar and drought treatments and highly inducible genes also exist in each specific stress treatment. Considerable progress in our understanding of the functions of ASR in plants has been made in the past two decades. However, the intrinsic lack of structure of ASR and the mechanism by which LLA23 communicates between the cytoplasm and the nucleus coupled with the occurrence of masking processes that allow LLA23 to remain in the cytoplasm should warrant further investigation.

Gene expression is organized in a hierarchy, the top of which is governed by a regulatory transcription factor. Many transcription factors govern the expression of stress-responsive genes, either cooperatively or independently, in controlling plant growth upon various stresses. ASR functions as a functional protein (osmoprotectant) and a regulator of many important processes, such as signaling and hormone responses, carbohydrate and protein metabolism, cytoskeletal organization, and defense and abiotic responses. Importantly, these advances would help clarify the organization of poorly understood signaling network and sugar-based regulation and coordination of carbohydrate and protein metabolism in plants. Clearly, more of these signaling partners need to be isolated and investigated to uncover the multiple regulatory layers in each signaling pathway, including the targeting components of ASR at the cellular and subcellular levels, and the various amplifications of the signal through different effectors.

Our understanding of the molecular mechanisms that underlie various abiotic stresses has improved considerably because of the development of advanced technologies that allow the study of transcription factor function on a genome-wide scale. The results obtained from our microarray analysis paved the way for more thorough investigations into the molecular mechanisms underlying drought-, sugar-, and ASR-induced signaling. However, as with all microarray analyses, the interpretation of the transcript changes requires caution because the changes in mRNA levels may not correlate with the changes in protein or enzyme activity levels. Furthermore, although microarray analysis is sensitive in signal detection, it only provides a transient snapshot of gene expression.

The threat of climate change has become more apparent in recent years. Drought and temperature stresses are the two major abiotic constraints imminently faced by sessile plants. The elucidation of drought and temperature resistance mechanisms in plants is therefore an important issue. Evidence has shown that ASR efficiently protects plants against drought, heat, and low-temperature stresses. These advantages should be applied to improve crops and economically important plants against various environmental stresses.

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CHAPTER THREE

Mechanisms Underlying the Initiation and Dynamics of Neuronal Filopodia: From Neurite Formation to Synaptogenesis

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Abstract

Filopodia are finger-like cellular protrusions found throughout the metazoan kingdom and perform fundamental cellular functions during development and cell migration. Neurons exhibit a wide variety of extremely complex morphologies. In the nervous system, filopodia underlie many major morphogenetic events. Filopodia have roles spanning the initiation and guidance of neuronal processes, axons and dendrites to the formation of synaptic connections. This chapter addresses the mechanisms of the formation and dynamics of neuronal filopodia. Some of the major lessons learned from the study of neuronal filopodia are (1) there are multiple mechanisms that can regulate filopodia in a context-dependent manner, (2) that filopodia are specialized subcellular domains, (3) that filopodia exhibit dynamic membrane recycling which also controls aspects of filopodial dynamics, (4) that neuronal filopodia contain machinery for the orchestration of the actin and microtubule cytoskeleton, and (5) localized protein synthesis contributes to neuronal filopodial dynamics.

1. INTRODUCTION

Actin is one of the most highly conserved and ancient proteins in nature. In eukaryotes, along with many associated proteins that regulate its function, the actin cytoskeleton accounts for up to 10–20% of cellular protein. Actin is monomeric in solution but through de novo nucleation and polymerization forms actin filaments. Actin filaments have fundamental roles in cellular morphogenesis. The evolutionary origins of actin are ancient and date back to the evolution of bacteria exhibiting cellular morphologies that deviate from a spherical phenotype (Cabeen and Jacobs-Wagner, 2010), underscoring the importance of actin in the regulation of cellular morphology. Actin filaments form a submembranous cytoskeleton that determines cell shape and is required for the outward protrusion and maintenance of the cell edges. The major protrusive structures formed by actin filaments are lamellipodia and filopodia (Fig. 3.1A). These protrusive structures allow the cell to probe and sense its environment, and also accordingly alter its shape and motility. This chapter reviews the cellular mechanisms that give rise to filopodia, focusing on lessons learned from the study of neurons.

Neuronal filopodia are dynamic finger-like projections extending outward from the surface of cells (Fig. 3.1B). They are usually initiated as small nubs protruding from the cell surface. The nub subsequently elongates giving rise to a shaft. Neuronal filopodia generally attain maximal lengths in the range of 5–10 μm, but can occasionally extend up to tens of micrometers from the site of initiation. The width of filopodia is on the range of 100–900 nm. Filopodia are dynamic and can exhibit a variety of behaviors including lateral bending of the entire filopodium and bending at hinge

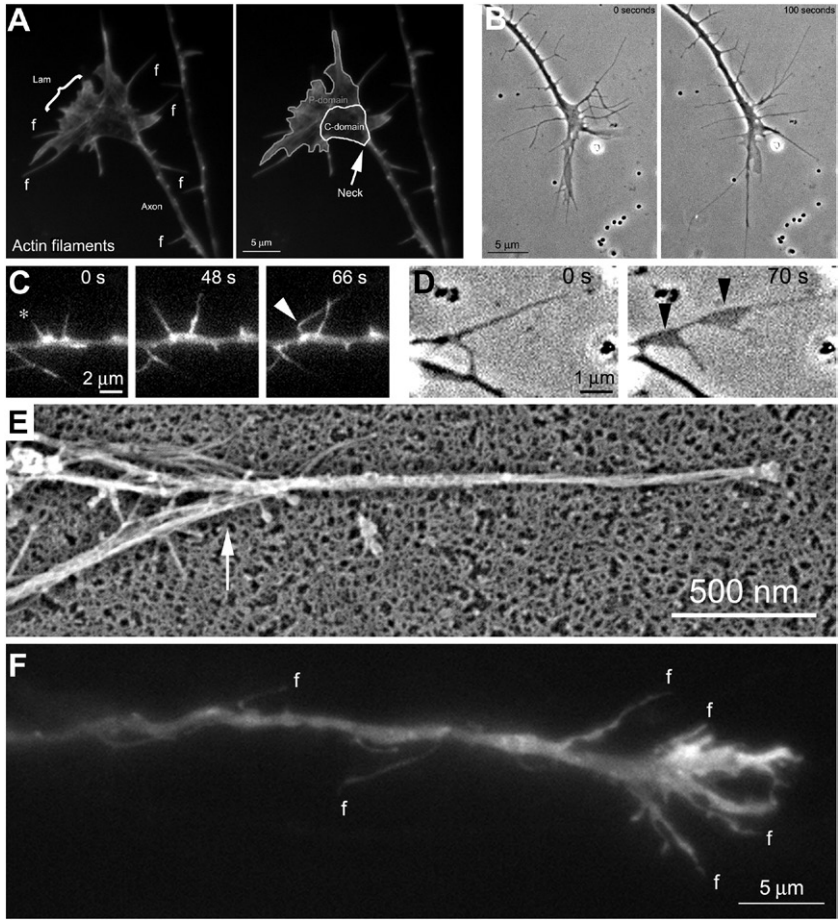

Figure 3.1 Neuronal filopodia. Panels A–E show primary chicken embryo sensory neurons in vitro and panel F shows an example of a sensory axon in the living embryonic spinal cord. (A) Growth cones are described as consisting of a peripheral (P) and central (C) domain. The P-domain is characterized by lamellipodia (lam) and filopodia (f). The growth cone "neck" is denoted by the arrow and represents the site of axon consolidation reflective of suppression of protrusive activity and formation of the axon shaft proper. The sample was stained with phalloidin to specifically reveal actin filaments and not soluble actin. (B) Two stills form a phase contrast timelapse sequence. Note that during the 100 s period, the growth cone largely changes its shape and the distribution of filopodia and lamellipodia. (C) Timelapse sequence from an axon expressing eYFP-β-actin. Note that the filopodium denoted by the * initially extend outward (48 s) and the buckles near its base (66 s, arrowhead). (D) Two stills from a phase contrast timelapse sequence showing the emergence of lamellipodium from the shaft of a filopodium (arrowheads). (E) Platinum replica electron micrograph of the actin cytoskeleton of an axonal filopodium (collaborative work with Dr T. Svitkina, University of Pennsylvania). Note that the actin filaments at the base of the filopodium become bundled together (arrow) and form the shaft of the filopodium. (F) Example of an axon extending in the living embryonic spinal cord. RFP-cortactin was expressed in sensory neurons through *in ovo* electroporation and imaged live following acute dissection of the living spinal cord (Spillane et al., 2011). Note the presence of filopodia (f) along the axon shaft and at the growth cone.

points along their shafts (Fig. 3.1C). The shafts of filopodia can also give rise to new lamellipodia or filopodia (Fig. 3.1D, also see Fig. 3.2A,D). Most filopodia are transient and are retracted back into the cell following their initial protrusion. An invariant and defining feature of filopodia is that they are dependent on the actin filament cytoskeleton. Specifically, the filopodium is supported by a bundled array of actin filaments (Fig. 3.1E). In neurons, filopodia are found along axons and dendrites, and also at the growth cone, both in vitro and in vivo (Fig. 3.1F). Actin filaments have fast growing "barbed ends" and less dynamic "pointed ends." In filopodia, as a general rule, actin filaments have their barbed ends directed toward the tip of the filopodium, while the pointed ends of the filaments are located at the base of the filopodium, or deeper into the cell. Actin filaments underlying cellular protrusions are highly dynamic with half-lives ranging from seconds to minutes. However, actin filaments in neuronal growth cone filopodia have markedly longer half-lives (20–25 min; Mallavarapu and Mitchison, 1999).

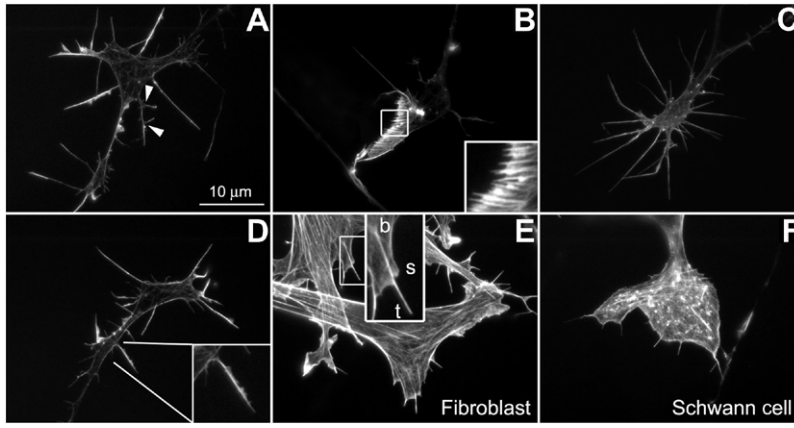

Figure 3.2 Examples of in vitro sensory axons (A–D) and nonneuronal cells (E–F) stained with phalloidin to reveal actin filaments. The arrowheads in (A) denote a filopodium that gave rise to additional filopodia from its shaft. The inset in panel (D) shows a filopodium that has given rise to a small lamellipodium along most of its length. While most of the filopodia in panels (A), (C) and (D) arise directly from the leading edge of the growth cones, panel (B) shows an example of filopodial "ribs," the tips of which extend only a short distance from the leading edge and the shaft/rib of the filopodium is embedded in a lamellipodium (see inset for higher magnification). Panel (E) shows a fibroblast and the inset denotes, as in panel (B), a filopodium with its base (b) and a shaft/rib (s) that is largely located within the cytoplasm of the cell, and its tip (t) projecting outward. Panel (F) shows the leading lamellipodium of a peripheral nervous system glial cell termed a Schwann cell. These cells have the same developmental origin as sensory neurons (neural crest) but clearly different morphology and functions.

Actin filaments used to build filopodia are nucleated *de novo* from the pool of cytoplasmic monomeric actin, a task accomplished by multiple families of actin nucleation factors discussed in Section 4.2. Additional proteins regulate the rate of polymerization, stability and depolymerization of filaments following nucleation, discussed in Section 4.3. Importantly, the mere availability of filaments is insufficient for proper cellular physiology. Therefore, cells utilize a large number of proteins to generate specific structures using actin filaments, further discussed in Section 4.2 (Michelot and Drubin, 2011).

Most, if not all, metazoan cells can form filopodia. Indeed, filopodia have fundamental roles throughout the life cycle of metazoans. As a few examples, during early embryonic development, filopodia have roles in cell–cell contacts and communication during blastulation and grastrulation (Nakatsuji, 1974; Satoh, 1978). Similarly, filopodia serve to anchor cells from different germ layers in the primitive streak of both rat and chicken embryos (Solursh and Revel, 1978). The filopodia of endothelial cells likely have roles in sprouting angiogenesis (De Smet et al., 2009; Eilken and Adamns, 2010). In cancer, filopodia serve as invasive structures promoting metastasis (Machesky, 2010). Atlhough filopodia are prominent features of many cell types, this review focuses on primary neurons in vivo and in vitro. Neurons exhibit the most complex and polarized morphology of any metazoan cell, and the morphogenesis of neurons is completely dependent on the actin and microtubule cytoskeleton. As detailed in Section 2, due to the many varied function of filopodia in neurons, the neuron is an excellent model system for addressing a multitude of questions regarding the biology of filopodia. Section 2 of this chapter emphasizes the varied roles of filopodia in the biology of neurons, and it is intended as a primer for readers not familiar with neuroscience. Sections 3–5 focus on the cellular mechanisms underlying the regulation of neuronal filopodia, and the mechanisms that link actin-filament-based filopodia to microtubules.

2. ROLES OF FILOPODIA IN THE DEVELOPMENT OF NEURONAL MORPHOGENESIS AND CIRCUIT FORMATION

2.1. Initiation of Axons and Dendrites

Following exit from the mitotic cycle in the developing neural tube, differentiated central nervous system neurons become postmitotic and migrate through the developing nervous system to find their appropriate location

(Faux et al., 2012). Peripheral nervous system neurons develop from migrating neural crest cells, or from placodes that form in situ within a tissue. Neural crest cells arise from the dorsal neural tube and migrate throughout the embryo and give rise to both neurons and nonneuronal cells. Sensory ganglia are the structures that allow the central nervous system to receive sensory information and also mediate proprioception. Neural crest cells converge and form cell aggregates which subsequently differentiate into a variety of sensory neurons and ganglia. Migration of central nervous system neuronal cell bodies and neural crest cells depends on the establishment of polarized leading edges, which serve as the machinery for sensing and responding to the extracellular environment (Krispin et al., 2010; Manent et al., 2011). Central nervous system neurons form dendrites and axons, while some peripheral nervous system neurons (e.g. dorsal root ganglion neurons) only form axons. In the case of central nervous system neurons, the cell body initially extends multiple "minor processes," which are undifferentiated and cannot be parsed into axons or dendrites. However, following the extension of these minor processes, the neuron selects one for differentiation as the axon, while the rest differentiate into dendrites. The issue of the specification of the axon and dendrites from the multiple undifferentiated minor processes formed by neurons undergoing morphogenesis is not discussed here, and the reader is directed to previous reviews of the topic (Cheng and Poo, 2012).

The leading edge of neuronal processes starts as a lamellipodial protrusion from the neuronal cell body and exhibits filopodia, or in some cases, filopodia form without lamellipodia. In vitro studies of the initial stages of protrusion have unveiled fundamental aspects of the cytoskeletal machinery of the early stages of axon and dendrite formation from cell body protrusions. The formed axon and dendrites are dependent on both actin filaments and microtubules for their proper function. Similarly, both actin filaments and microtubules contribute to the emergence of axons and dendrites. The regulation of cytoskeletal dynamics in filopodia extending from neuronal cell bodies has emerged as one of the earliest steps in the formation of axons and dendrites and will be discussed further. Insights into the formation of filopodia during preaxon/dendrite formation stages of development, and the maturation of filopodia into neuronal processes, were obtained through careful analysis of the process using a variety of imaging modalities (Smith, 1994a,b). Differential interference contrast imaging revealed that an early step in the formation of neuronal processes is the invasion of the filopodium by cytoplasm, which causes the filopodium to swell and increase in

diameter. Microtubules serve as the major tracks for the transport of organelles and proteins in developed axons and dendrites, and likely have a similar role during the initial stages of process formation. Indeed, both the redistribution of stabilized microtubules and the entry of microtubules through dynamic instability into the protrusions of early stage neurons contribute to the maturation of these protrusions into processes depending on the neuronal type (Smith, 1994b; Dent et al., 2007). Interestingly, a similar dependence on microtubule redistribution or dynamic instability has been reported for the *de novo* formation of axon branches from preexisting axons (Gallo and Letourneau, 1999; Dent and Kalil, 2001), which may share similarities with the initial formation of the axon from the cell body. Regardless of whether microtubules are targeted to filopodia through redistribution or dynamic instability, collectively, these studies identify the penetration of filopodia by microtubules as a fundamental step in the formation of axons and dendrites.

Studies of the molecular mechanism that coordinates the actin filament and microtubule cytoskeleton during minor process formation have identified molecules that link the two cytoskeletal systems. MAP2c is a microtubule associated protein, which, however, can also bind and crosslink actin filaments into bundles, as characteristically found in filopodia (Roger et al., 2004). MAP2c targets to nascent neuronal processes and expression of MAP2c mutants that fail to bind microtubules, or protein kinase A, impairs process formation in hippocampal neurons (Dehmelt et al., 2003). Additional studies have shown that the actin filament binding property of MAP2c is also required for MAP2c to promote process formation (Roger et al., 2004), indicating that it may act to physically link microtubules and actin filaments. Drebrin is an actin-filament-binding protein that is highly expressed in brain tissue (Dun and Chilton, 2010). Drebrin regulates the actin cytoskeleton by binding to filaments and altering the ability of other proteins to bind the filaments. However, drebrin also binds EB3 (Geraldo et al., 2008; Bazellières et al., 2012), a protein that specifically binds the + tips of microtubules when they are polymerizing. The interaction between EB3 and drebrin may serve to target microtubule tips into filopodia during early stages of process formation (Geraldo et al., 2008). While much remains to be learned about how microtubules and actin filaments are coordinated during early process formation, these studies point to the merging theme that molecules which bind both cytoskeletal components, directly or indirectly, are a major regulatory point.

Regulation of actin filaments underlies the initial formation of filopodia, and subsequent minor process extension, from neuronal cell bodies. The

Arp2/3 complex is an actin nucleating complex that binds to the sides of existing actin filaments and nucleates a new filament at approximately a 70° angle from the original filament (Yang and Svitkina, 2011). Arp2/3 activity thus results in a branched network of actin filaments and it is generally considered to mediate lamellipodial protrusion. However, a role for the Arp2/3 complex-mediated branched network has also been proposed in the formation of filopodia (Yang and Svitkina, 2011; discussed further in Section 4.2). The role of the Arp2/3 complex in the formation of minor processes, axons and dendrites from neuronal cell bodies is not clear. Inhibition of Arp2/3 complex function through siRNA-mediated knock down of two complex subunits (Arp3 and p34-Arc) in cultured hippocampal neurons did not inhibit minor process formation, but rather it resulted in increased numbers of minor process which, however, extended aberrantly (Korobova and Svitkina, 2008). In a separate study, also using cultured hippocampal neurons, the Arp2/3 complex was inhibited through the expression of a peptide that prevents activation of the complex, which otherwise exhibits minimal baseline activity in the absence of extracellular signals activating it (Strasser et al., 2004). In these experiments, inhibition of Apr2/3 complex function had no effect on either axon formation or the number of dendrites per neuron. Collectively, these studies indicate that the Apr2/3 complex has a minimal, if any, role in the formation of minor processes from neuronal cell bodies.

Ena/VASP proteins are major regulators of actin filament barbed end polymerization. One mechanism of action of Ena/VASP proteins is to act as anticapping proteins. If the barbed end of a filament is capped by capping proteins, its polymerization will be impaired (Menna et al., 2011). Ena/VASP proteins thus may serve a role as local regulators of the capping of filaments in discrete cellular domains. However, it is noteworthy that Ena/VASP proteins may have additional functions to anticapping, possibly including inhibition of Arp2/3-mediated filament branching, bundling of actin filaments and other more controversial functions (Bear and Gertler, 2009). Genetic deletion of Ena/VASP proteins in mice results in multiple defects in brain cortical development including ectopias, improper layering of the cortex and failure to form some major axon tracts (Kwiatkowski et al., 2007). In vitro analysis of minor process formation from Ena/VASP deleted neurons provided insights into the role of these proteins in the formation of filopodia, and revealed an unexpected complexity in the mechanisms that can regulate filopodia formation from neuronal cell bodies (Dent et al., 2007). When cortical neurons lacking Ena/VASP were plated on a polylysine substratum, they exhibited a striking deficit in minor process formation

and were characterized by broad circumferential lamellipodia devoid of filopodia. Live imaging of minor process formation in wild-type neurons revealed that consistent with previous studies, these processes are initiated by filopodial structures. In a few cases where Ena/VASP deleted neurons formed processes, this also occurred through the thickening and subsequent elongation of a single filopodium. Interestingly, the Ena/VASP deletion phenotype could be bypassed to different degrees by culturing the neurons on laminin, meningeal fibroblasts, or through the expression of other proteins previously shown to promote filopodia formation (e.g. myosin X and the formin mDia2) or inhibition of myosin II (also see Kollins et al., 2009). Thus, collectively, the data indicate that the neuronal cell body may have multiple mechanisms that can be utilized to generate filopodia-dependent minor processes in an environment-dependent manner.

A recent study has determined a role for F-BAR proteins in the regulation of minor process formation (Saengsawang et al., 2012). F-BAR proteins have been involved in regulating the local degree of membrane curvature during endocytosis and the formation of filopodia (Suetsugu et al., 2010). Genetic deletion of the F-BAR Cdc42-interacting protein 4 (CIP4) promoted the rate of minor process development by cortical neurons. Conversely, overexpression of the protein impaired process formation and resulted in prominent lamellipodial formation from neuronal cell bodies. Unlike in nonneuronal cells, the effects of CIP4 on neuronal process formation are mediated through regulation of lamellipodia and not endocytosis or filopodia, indicating a neuron-specific role for CIP4.

2.2. Extension and Guidance of Axons and Dendrites

Following the emergence of minor processes from the neuronal cell body, the neuron differentiates one of these processes into an axon, and the rest develop into dendrites. Axons and dendrites differ in significant ways. The organization of microtubules is a prominent distinction between axons and dendrites. Within axons, most microtubules have their fast growing + end directed toward the distal tip of the axon. In contrast, in dendrites, the orientation of microtubule + ends is almost equally distal and proximal. Additionally, other cytological and molecular differences distinguish axons and dendrites, but are not discussed here. For the purposes of this review, it is relevant to keep in mind that axons and dendrites are different cellular domains, and specific differences will be discussed on a per need basis.

During development, dendrites extend and branch in order to form a dendritic field. Dendrites are usually in the order of a few 100 micrometers

in length at most, serve as the "antennae" of neurons, and establish synaptic contacts with incoming axons. In contrast, axons have to extend relatively long distances throughout the developing embryo and must be properly targeted to the correct nervous system region in order to establish synaptic contacts with the dendrites of appropriate target neurons. The process by which extracellular signals determine the direction of the extension of axons is termed *axon guidance/path finding*. Axon elongation and guidance is mediated by a specialized motile structure located at the tip of the axon termed *the growth cone* (Fig. 3.1A). Axonal growth cones are characterized by two major domains, the peripheral (P) and central (C) domain. The P-domain consists of lamellipodia and filopodia. The C-domain is the interface between the end of the axon shaft proper and the P-domain (Fig. 3.1A). The C-domain contains few actin filaments, relative to the P-domain, but is enriched in the + tips of microtubules. Just proximal to the C-domain, protrusive activity is shut down giving rise to a mostly quiescent axon shaft and the transition between the dynamic growth cone and the axon shaft is termed *the growth cone neck* (Fig. 3.1A). Extending dendrites also have growth-cone-like structures at their tip, but have received much less attention than axonal growth cones. The growth cones of extending axons interact with soluble signals, the surfaces of other cells and the extracellular matrix and interpret these extracellular signals in order to determine the direction of process extension. The guidance of neuronal processes is thus driven by extracellular signals, which result in changes in signaling pathways within the growth cone, ultimately leading to cytoskeletal reorganization and redirection of the extending growth cone.

The extension of established axons proceeds through a series of growth cone cytoskeletal and morphologic rearrangements, which can be broken into three distinct phases (Dent and Gertler, 2003). The first phase is termed *protrusion* and involves the P-domain. During this phase, the growth cone extends actin-filament-dependent lamellipodia and/or filopodia. The second phase is termed *engorgement* and involves the C-domain. Engorgement is a microtubule-based phase of extension and refers to the movement of microtubule tips into recently protruded domains of the growth cone. The forward advance of microtubule tips can occur through microtubule + end polymerization or, to some degree, the transport of subsets of axonal microtubules. Microtubules engage actin filaments in the P-domain, and the retrograde flow of actin filaments from the P-domain toward the C-domain serves to prevent microtubules from extending forward (Schaefer et al., 2002, 2008). Inhibition of myosin II, which mediates retrograde flow, allows

microtubules to penetrate into the P-domain thereby promoting engorgement (discussed further in Section 5.2). The final phase is termed *consolidation* and occurs at the growth cone neck (Fig. 3.1A). Consolidation is characterized by the sharp decline in protrusive activity as the growth cone transitions into the axon shaft. As noted above, the growth cone is a highly polarized structure and consolidation is required to keep the growth cone polarized at the tip of the extending axon. The axon advances through sequential reiteration of these three phases, while also maintaining a polarized growth cone at its tip. Guidance is thought to occur through the regulation of all three phases of axon extension. Through the spatiotemporal control of the protrusion, engorgement and consolidation phases, extracellular signals can redirect the extension of the axon.

A role for filopodia in the regulation of the rate of axon extension rate (i.e. distance/unit time), independent of growth cone guidance, has not been unequivocally described. Indeed, depending on the substratum neurons are cultured on (Abosch and Lagenaur, 1993), or the developmental age of the neurons (Jones et al., 2006), axons can extend at intermediate to normal rates in the absence of actin filaments all together, sometimes even at faster rates (Ruthel and Hollenbeck, 2000). However, the extension of the axon is strictly dependent on the forward advance of microtubules during engorgement, regardless of whether actin filaments are present or not (Letourneau et al., 1987; Jones et al., 2006). In contrast, a requirement for actin filaments in the guidance of axons is well established. In vitro and in vivo studies revealed a fundamental role for actin filaments, and presumably filopodia, in the ability of axons to respond to extracellular cues (Dent and Gertler, 2003). In vitro contact of a single filopodium with a guidance cue is sufficient to reorient the growth cone, and thus change the direction of axon extension (Gomez and Letourneau, 1994; Gallo et al., 1997). Similarly, in order for an axon to extend over a nonpermissive region of the substratum in vitro (e.g. a nonpermissive stripe between two permissive regions), the filopodia of the growth cone must be able to reach across the nonpermissive region to contact a permissive region and extend over the nonpermissive region (Hammarback and Letourneau, 1986). Live imaging studies of growth cone guidance in response to contact with "guidepost" cells in the limb buds of grasshopper embryos have revealed that this guidance is mediated by contact of single filopodia with the guide-post cells (O'Connor and Bentley, 1993; discussed further in Section 5). While undergoing guidance to their targets in vivo, axonal growth cones exhibit a striking variety of morphologies, depending on context. As a rule-of-thumb, the growth cones

of axons extending linearly (e.g. on top of previously guided axons) tend to have relatively simple morphologies characterized by few filopodia and lamellae. In contrast, when growth cones arrive at regions of the developing nervous system where their trajectory must change significantly, they often develop more complex morphologies (Tosney and Landmesser, 1985). The elaboration of growth cone morphology at these "decision points" may represent an attempt by the growth cone to increase its ability to sample the environment and arrive at a guidance decision.

Although a role for filopodia in growth cone guidance has been supported by in vitro and in vivo studies, a study investigating the effects of inhibiting Ena/VASP protein function in developing *Xenopus* retinal ganglion cell axons in vivo has indicated that filopodia may not be obligatory (Dwivedy et al., 2007). In *Xenopus*, retinal ganglion cells project their axons into the developing optic tectum. Along the path, the growth cone has to make guidance decisions at multiple points. Surprisingly, depletion of Ena/VASP proteins, which decreases the mean number of growth cone filopodia by 70–90% depending on the location of the growth cone along its path to the tectum, did not impair the ability of axons to ultimately arrive at their target without making overt guidance errors (i.e. projecting to inappropriate regions along their path). However, the net rate of axon advance was decreased at multiple points along the path to the tectum, and the decrease was due to increased time spent stalling without net advance. While one interpretation of these results is that growth cones with greatly diminished numbers of filopodia, but not completely devoid of filopodia as noted above, can ultimately pathfind correctly, an alternative interpretation could also be considered. As noted previously, growth cones often stall at "decision" points along their path and elaborate filopodia and lamellipodia. Presumably, this increase in growth cone morphologic complexity reflects periods during which the growth cone is sampling its environment using protrusive structures in order to parse the guidance information in its environment. Therefore, when the number of growth cone filopodia is decreased, but not abolished, by Ena/VASP knock down, growth cones may be taking more time to make filopodia-dependent guidance decisions (as noted in Dwivedy et al., 2007). Indeed, Ena/Vasp depletion increased the stalling of growth cones in vivo. Determination of the independence of growth cone guidance from filopodia would therefore benefit from additional investigations in which *all* filopodia are abolished at growth cone, perhaps through concerted depletion of multiple pathways that mediate filopodia formation. The depletion of Ena/VASP did not abolish lamellipodia formation at

growth cones, and in vitro lamellipodia can mediate guidance decision in the absence of filopodial contact with a localized guidance cue (Gallo et al., 1997). Thus, lamellipodia may be able to compensate for filopodia during growth cone guidance. Nonetheless, the intriguing study by Dwivedy et al. (2007) provides exciting data and indicates that further analysis of the role of filopodia in axon guidance is warranted.

Some neuronal circuits exhibit the property of dendritic tiling (Gao, 2007). *Tiling* refers to the formation of spatially nonoverlapping fields of dendritic arborization by neurons with shared functions within the circuit, or more generally, the formation of nonoverlapping dendritic fields between adjacent neurons. Tiling arises through contact-mediated repulsion between nascent dendrites and is mediated by multiple cell surface signals on the membranes of dendrites (e.g. dsCAMs; Hattori et al., 2008). Similarly, dendrites from the same neuron can also exhibit tiling and form nonoverlapping fields. The repulsion between dendrites is mediated by contacts initiated by dendritic filopodia in vivo (Baker and Macagno, 2007). Similarly, the repulsion of axonal growth cones induced by repellent signals (e.g. semaphorins) can be mediated by contact of a single filopodium with a point source of the repellent (Fan and Raper, 1995) of the surface of a cell with repellent properties (Oakley and Tosney, 1993). Thus, neuronal filopodia serve as receptor structures for both attractant and repellent signals.

2.3. Formation of Axon Collateral Branches and Dendrite Branches

Although consolidation (Section 2.2) greatly decreases protrusive activity along the axon shaft proximal to the growth cone neck, filopodia can also form along the axons shaft. These axonal filopodia have a fundamental role in neurodevelopment, the initiation of axon collateral/interstitial branches (Dwivedy et al., 2007; Gallo, 2011). The formation of collateral branches is required for the proper development of neuronal circuitry and is considered to be a major mechanism underlying the ability of the injured nervous system to rewire damaged circuitry (Fawcett, 2009; Gibson and Ma, 2011; Onifer et al., 2011). During development, neurons form one axon but need to establish synaptic connections with up to thousands of other neurons. In order to achieve this high order of connectivity, the single axon generates numerous side branches each contributing to the establishment of subsets of the final set of synapses formed by the neuron. Collateral branching allows a single neuron to connect to disparate regions of the nervous system and/or cover a large extent of territory within a single target region. Thus,

understanding the formation of collateral branches is a fundamental issue in neuroscience.

In vivo and in vitro axon collateral branches are initiated as axonal filopodia (Gallo, 2011). Axons generate many transient filopodia, a subset of which matures into collateral branches. Similar to the main axon shaft, collateral branches are supported by a microtubule array. The entry of axonal microtubules into axonal filopodia is considered to be fundamental to be ability of the filopodium to mature into a branch. The formation of axonal filopodia is under control by target tissue derived signals. The regulation of axonal filopodial formation and dynamics has been most intensively studied in the context of the branching of retinal axons and cortical axons. For the purpose of this review and didactics, I will focus on the development of branches along cortical axons projecting into the spinal cord (O'Leary et al., 1990). During development, cortical neurons project axons into the spinal cord. The axons initially grow past the developing pons. As the pons develops, the segments of axons adjacent to the pons begin to exhibit higher rates of axonal filopodia formation ultimately culminating in the maturation of a subset of these filopodia into collateral branches that extend into the pons (Bastmeyer and O'Leary, 1996). In vitro coculture studies using explants of pons tissue have determined that the pons releases soluble factors, which promote the formation of axonal filopodia and branches (Sato et al., 1994). It is also noteworthy that the process of branching is not simply controlled by factors that promote to formation of axonal filopodia and branches, but also by factors that inhibit the formation of branches or promote their retraction (Gibson and Ma, 2011; Gallo, 2011). Thus, a balance of branch promoting and inhibitory factors ultimately sculpts the architecture of axons.

Direct evidence for the dependence of branching on axonal filopodia was presented in the previously discussed study by Dwivedy et al. (2007; Section 2.2). A major finding of this study was that although axons eventually found their path to their synaptic target regions, the optic tecum, when they arrived, the axons exhibited a near-complete failure to generate axonal filopodia and collateral branches. These exciting in vivo findings are consistent with many in vitro studies addressing the role of axonal filopodia in branching. This study also underscores the necessary role of axonal filopodia in the process of branching.

Similar to axons, dendrites form branches from filopodial precursors in vivo. Contact of a dendritic filopodium with an appropriate synaptic partner axon initiates calcium-based mechanisms leading to the formation

of postsynaptic structures and the maturation of the filopodium into a synapse bearing branchlet (Heiman and Shaham, 2010). One of the hallmark molecules of a functional postsynaptic structure is the postsynaptic density protein PSD-95, which acts as a scaffold for many other synaptic components. Brain derived neurotrophic factor (BDNF) is an important regulator of dendritic development (Horch, 2004). Accumulation of PSD-95 in dendritic filopodia is regulated by BDNF signaling. Interestingly, BDNF promotes the targeting of PSD-95 through regulation of the entry of dendritic microtubule tips into maturing dendritic filopodia (Hu et al., 2011). Cypin is an additional link between dendritic filopodia, PSD-95 and the regulation of microtubules during maturation of the filopodium into a branch (Firestein et al., 1999). Cypin is a guanine deaminase that binds PSD-95 and promotes microtubule polymerization and dendritic branching (Georges et al., 2008). Furthermore, PSD-95 also interacts with microtubule end-binding protein 3 (EB3) and adenomatous polyposis coli (APC) and these interactions have been suggested to inhibit and promote dendrite branching, respectively (Georges et al., 2008). These studies link regulation of microtubule entry into dendritic filopodia and the process of dendritic branching. However, a complete analysis of the cytoskeletal mechanisms of dendritic branching is lacking.

2.4. Formation of Synaptic Structures

Functional chemical synapses represent contacts between the membranes of the pre- and postsynaptic neurons. The mature chemical synapse consists of a postsynaptic side and a presynaptic active zone, and a synaptic cleft separating the two. In most cases, both structures are in the order of one to a few micrometers. Neurotransmitters are released through the exocytosis of vesicles, present in the presynaptic active zone, into the synaptic cleft where they bind receptors and activate signaling pathways in the postsynaptic button. The formation of dendritic filopodia is well established as one for the first steps in the process of synaptogenesis (Menna et al., 2011). Following contact with the presynaptic neurons, the dendritic filopodia maturate into structures termed *spines*. Similarly, the filopodia of axons may serve as the presynaptic structures that mature into active zones. Consistent with this notion, highly dynamic axonal filopodia/branch protrusions contain synaptic vesicle release machinery (Pinches and Cline, 1998). However, a recent detailed live imaging and electron microscopy correlative study, while confirming that dendritic filopodia are precursors to postsynaptic structures, has revealed that functional presynaptic structures in the developing

visual system of *Xenopus* arise mainly from stable axon branches and not from dynamic axonal filopodia (P.P. Li et al., 2011). As discussed above, axon branches arise from axonal filopodia, but axonal filopodia may be a step removed from the mechanisms that give rise to presynaptic structures. The mechanisms of the formation of axonal and dendritic filopodia and branches are discussed in detail in the subsequent sections of this chapter. The maturation of a dendritic filopodium into a spine is not discussed and has been covered extensively elsewhere (Chapleau et al., 2009).

3. MOLECULAR AND ULTRASTRUCTURAL ORGANIZATION OF FILOPODIA

3.1. Filopodia: Identical Twins or Cousins?

The term *filopodium* is generally used to refer to a linear cellular protrusion that undergoes tip-mediated elongation. Filopodia are considered to be structures supported by a bundle of actin filaments with the rapidly polymerizing barbed ends pointed toward the tip of the elongating filopodium. However, it is far from clear whether all cellular protrusions that fit the above definition of a filopodium are generated by identical mechanisms. Minimally, three basic events are required for the formation of a filopodium: the nucleation of actin filaments, the elongation of actin filaments through barbed end polymerization, and the orchestration of the filaments into a polarized bundle. In cells, the nucleation, polymerization and organization of actin filaments can be regulated by multiple molecular systems. For example, there are many actin filament nucleating system (e.g. formins, Arp2/3, spire, cordon bleu; Firat-Karalar and Welch, 2011) and multiple proteins that can organize filaments into bundles (Winder, 2003). The general appearance of filopodia can differ between cell types, different cells in the same population, or even within the same cell. Figure 3.2 shows examples of the appearance of actin filaments in filopodia from a variety of cell types, as revealed by phalloidin labeling which specifically labels actin filaments. Structures generally termed filopodia can consist of actin bundles that form at the edge of the cellular perimeter and extend outward up to 10 μm or more (Fig. 3.2A,C), or have tips that only slightly protrude from the cellular perimeter but have long extended intracellular "ribs" (Fig. 3.2B,E). Thus, it is possible to imagine the formation of filopodia through different, but not necessarily completely distinct, molecular mechanisms, which, however, achieve the same end of generating a linear actin-filament-based cellular protrusion.

One model of filopodial formation proposes that actin filaments in preexisiting branched arrays generated by the Arp2/3 actin nucleating complex are recruited into filopodial bundles (Svitkina et al., 2003). However, examples of Arp2/3-independent formation of filopodia have been reported (Faix et al., 2009). Furthermore, the Arp2/3-based model was derived from studies of filopodia that emerge from lamellipodia, and prominent filopodia emerge from neuronal growth cones even when they are devoid of detectable lamellipodial protrusions (e.g. Fig. 3.2A,C,D). However, it cannot be ruled out that small nonprotrusive lamellipodial-like structures, similar to the axonal patches of actin filaments that give rise to axonal filopodia (Ketschek and Gallo, 2010; Spillane et al., 2011), may mediate formation of filopodia in the absence of large lamellipodia (see Goldberg et al., 2000 and Section 4.1). Similarly, consistent with the notion that filopodia arise from precursors structures, Steketee et al. (2001) provided evidence that both neuronal and nonneuronal filopodia emerge from cytological structures, which they termed *focal rings*. In contrast, another model for the formation of filopodia posits that the filaments used to generate the filopodium arise *de novo* through the action of other actin nucleating systems (e.g. formins) (Faix et al., 2009).

The GTPase Cdc42 is a major regulator of cellular physiology and was originally shown to drive increases in the number of filopodia in nonneuronal cells (Nobes and Hall, 1995). However, cases of Cdc42-independent formation of filopodia have been documented (Faix et al., 2009). For example, expression of dominant negative Cdc42 does not alter the number of spinal neuron growth cone filopodia on a polylysine substratum, but blocks the increase induced by addition of laminin (Brown et al., 2000), suggesting an extracellular context dependence of the requirement of Cdc42 in filopodia. Drebrin is an actin-filament-binding protein, mostly but not solely expressed in neurons, that can regulate the ability of other actin-binding proteins to associate with filaments (e.g. cofilin, myosin II; Dun and Chilton, 2010). Experimental expression of drebrin in cell lines that do not express drebrin increases the number of filopodia (Shirao et al., 1992), suggesting drebrin taps into an existing mechanism. In contrast, depletion of drebrin in neurons greatly blocks the formation of filopodia (Dun et al., 2012), indicating it is required for formation of filopodia in neurons but not in cells that do not normally express it. Similarly, expression of the neuron-specific proteins GAP-43 or synaptotagmin I leads to filopodia formation in nonneuronal cells (Zuber et al., 1989; Feany and Buckley, 1993). Thus, different cell types may utilize different sets of "molecular tools" to generate

filopodia, but the mechanism of filopodial formation can be tapped into in a manner relatively independent of the specific cell type, at least by some molecules.

A few studies have also suggested that the mechanisms underlying aspects of filopodia initiation, or development, differ at the subcellular level. For example, Meberg and Bamburg (2000) found that overexpression of actin depolymerizing factor in embryonic cortical rat neurons increases and decreases the number of filopodia at growth cones and along the axon shaft, respectively. In our own studies, we have found that introduction of dominant negative Rac1 into chicken sensory neurons decreases the number of both axonal and growth cone filopodia (Spillane et al., 2012). In contrast, introduction of dominant negative Cdc42 only decreased the number of growth cone filopodia without affecting the number of axonal filopodia in the same neurons. Consistent with this observation, a study investigating Cdc42 activity levels in axons and growth cones found high levels in the peripheral domain of growth cones, but minimal levels in the axon shaft (Myers et al., in press). Furthermore, the levels of Cdc42 activity detected in the axon were not affected by cell permeable peptide-mediated blockade of Cdc42, while the levels at the growth cone dropped to levels analogous to those detected in the axon. Similarly, the induction of filopodia by nerve growth factor and PI3K activation at the growth cone and along the axon shaft is independent and dependent on axonal protein synthesis, respectively (Spillane et al., 2012; also see Section 3.4). Dendritic filopodia also exhibit differences depending on whether they arise from the dendritic shaft or at the growth cone (Portera-Cailliau et al., 2003). Analysis of the developing dendrites of cortical neurons in slice cultures revealed that the motility, length and density of filopodia is greater at the growth cone than along the dendrite shaft, and manipulating neuronal synaptic activity differently affects shaft and growth cone filopodia. Thus, studies of neuronal axons, dendrites and growth cones have begun to unveil subcellular differences in aspects of the mechanisms underlying the regulation of filopodia.

An outstanding example of how filopodia can vary in structural organization was provided by a study comparing the ultrastructure of dendritic filopodia to growth cone and axonal filopodia using platinum replica electron microscopy (Korobova and Svitkina, 2010). In contrast to "conventional" filopodia, which exhibit a tightly bundled parallel array of actin filaments in their shaft, dendritic filopodia were found to contain a hybrid of long linear filaments with Arp2/3 complex branched filaments along the shaft. Furthermore, dendritic filopodia did not contain the actin bundling

protein fascin, which is otherwise found in filopodia, and due to its otherwise ubiquitous localization to filopodia, is even used as a filopodial "marker" (Dent et al., 2007).

Consideration of the studies discussed above suggests that filopodia may not be a unitary biological phenomenon, but rather as a final functional outcome that can be attained through multiple molecular mechanisms operating under the umbrella of a few general organization/mechanistic schemes. Indeed, recent studies of neuronal filopodia demonstrate that there are multiple molecular mechanisms that can orchestrate the formation of filopodia even in the same cell (Sections 2.1 and 3.2). Furthermore, as noted in a recent review (Michelot and Drubin, 2011), the nucleation mechanism of filaments can alter their physical properties and also the set of molecules that associate with filaments. As different filopodia may be derived from actin filaments nucleated by different nucleation mechanisms, which will impart different properties to the filaments, this may reflect the existence of functional subtypes of filopodia (e.g. axonal filopodia versus growth cone filopodia versus dendritic filopodia). Thus, it may be cautious to consider that although as a collective filopodia appear as closely related twins based on their phenomenology, they may be more akin to cousins belonging to a general family reflective of a cytoskeletal organizational scheme that can be tapped into through multiple molecular mechanisms, perhaps leading to functional differences.

3.2. Subfilopodial Localization of Proteins

The molecular composition of filopodia exhibits a striking degree of compartmentalization. At face value, the filopodium can be considered to be a "simple rod" made of parallel aligned actin filaments. However, structural and regulatory molecules are not uniformly distributed along this deceptively simple structure. This section addresses the molecular organization of neuronal filopodia.

As noted in Section 3.1, some filopodial filament bundles emerge directly from the cell's edge, while other exhibit "ribs" that localize many microns into the cytoplasm before giving rise to a usually short filopodial protrusion from the cell's edge. This section focuses on the former as they are the most commonly observed type and found in the growth cones of actively extending axons and axonal filopodia. Figure 3.3A shows an overview of the localization of a variety of molecules within neuronal filopodia, and a few examples of selected proteins (panels B and C). The list in this figure was generated based on reports of the localization of these proteins in either axonal growth cone or shaft filopodia. The list is not intended to imply

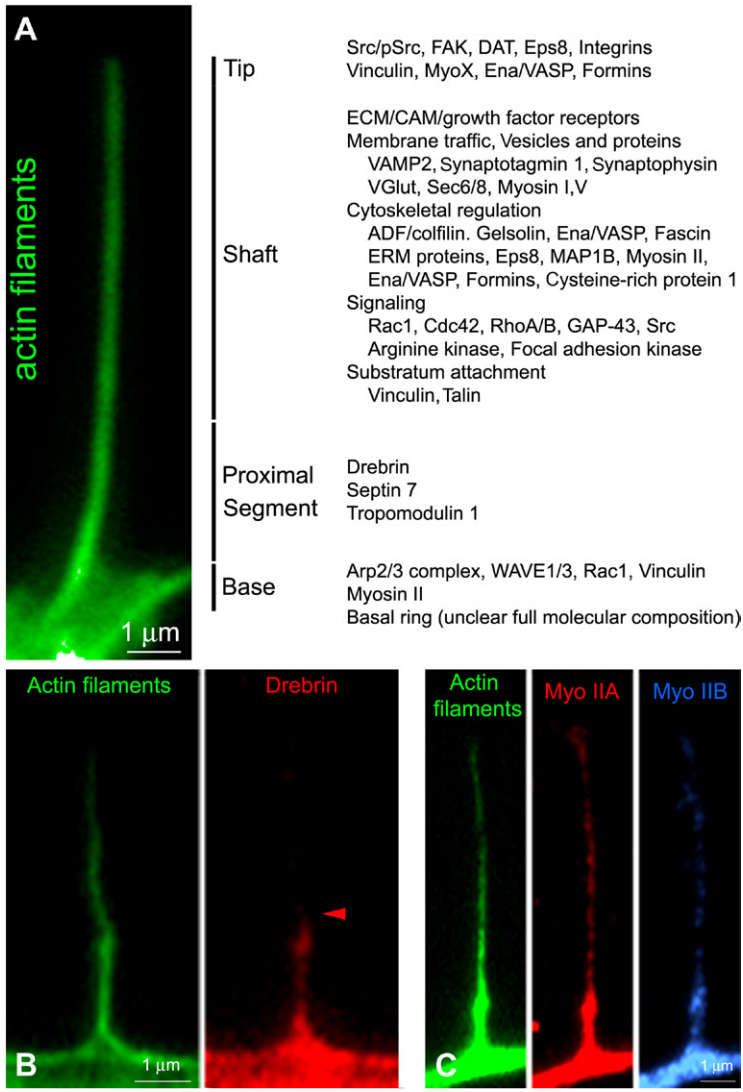

Figure 3.3 Subfilopodial compartmentalization. (A) Schematic showing the general subfilopodial localization of a variety of proteins found in filopodia. The filopodium is divided into three components: base, shaft and tip. The shaft is further divided into a proximal segment and the rest of the more distal shaft. References are provided in the text. (B) Example of antidrebrin staining in a filopodium counterstained with phalloidin to reveal actin filaments. Note that drebrin is largely confined to the proximal part of the shaft. The arrowhead denotes the distal extent of drebrin localization along the shaft. (C) Distribution of myosin IIA and IIB in a triple labeled filopodium. Puncta of both myosins are found throughout the filopodium. For color version of this figure, the reader is referred to the online version of this book.

that all filopodia will contain all listed proteins, but serves as a template for the general molecular organization of neuronal filopodia. For the purpose of this discussion, the filopodium is broken down into the following four subdomains: (i) the base of the filopodium, (ii) the proximal segment of the filopodium, (iii) the remainder of the distal filopodial shaft and (iv) the filopodial tip. Molecules representative of adhesion mechanisms, signal transduction apparatus, vesicle traffic, and regulation of actin filaments have all been localized within filopodia.

The base of the filopodium is the site where the filopodium originally formed. The mechanisms of the initiation of filopodia are discussed in Section 4.1. Analysis of the organization of actin filaments at the base of established neuronal filopodia using electron microscopic techniques has revealed that actin filaments undergo convergence into the filopodia shaft (Korobova and Svitkina, 2008, 2010; Spillane et al., 2011), consistent with the convergent elongation model (Svitkina et al., 2003). At the growth cone, the role of Arp2/3 in initiation of filopodia differs between cell types or in an extracellular context-dependent manner (Strasser et al., 2004; Korobova and Svitkina, 2008; Norris et al., 2009). In contrast, along the axon shaft, Arp2/3 is required for the formation of filopodia and collateral branches (Strasser et al., 2004; Spillane et al., 2011). In the case of axonal filopodia, regulators of the Arp2/3 complex have also been localized to the base of filopodia (e.g. WAVE1/3, cortactin; Spillane et al., 2011; Hu et al., 2012). Myosin II also targets to the base of filopodia, and inhibition of myosin II increases the number of filopodia both at the growth cone and along the axon shaft (Gehler et al., 2004; Loudon et al., 2006). Septins are a family of heterooligomeric proteins with various cellular functions, often linked to the regulation of the cytoskeleton (Mostowy and Cossart, 2012). Septin 6 binds actin filaments, preferentially at Arp2/3-mediated branches, and is found at the base of axonal and dendritic filopodia and in both cases, it is involved in the formation of branches (Cho et al., 2011; Hu et al., 2012). The base of growth cone filopodia also represents a site of substratum attachment and contains an ultrastructure termed the *basal ring* (Steketee et al., 2001). Unfortunately, little is known about the molecular composition of this intriguing structure. However, the Rac1 GTPase has been localized to rings (personal communication by Dr K. Tosney, University of Miami). Rac1 localizes to the actin patch precursors to the formation of axonal filopodia, and inhibition of Rac1 function decreases the number of filopodia both along the axon and at the growth cones of sensory neurons (Spillane et al., 2012).

The proximal 1/3–1/2 of the filopodial shaft of some neuronal filopodia appears to be a preferential targeting site for some actin-binding proteins. Drebrin is an actin-filament-binding molecule that in turn regulates the ability of other proteins to bind filaments (Dun and Chilton, 2010). In growth cone and axonal sensory neuron filopodia, the localization of drebrin is usually restricted at the proximal filopodial shaft (Fig. 3.3B). However, in the growth cones of cultured hippocampal neurons, drebrin does not appear to localize to filopodia, and in these growth cones, it accumulates at the interface between the peripheral and central domains (Mizui et al., 2009). These contrasting observations are from in vitro experiments, and it is not clear if they reflect a difference between neuron types, or regulation of drebrin localization by the substratum or growth factors. Septin 7 targets to the proximal shaft close to but not overlapping with the base of the filopodium (Hu et al., 2012; also see Fig. 3.7). A similar distribution of septin 7 has been reported for dendritic spines and filopodia (Tada et al., 2007). Interestingly, both drebrin and septin 7 have been shown to bind microtubules or microtubule-associated proteins (e.g. drebrin binds EB3; Geraldo et al., 2008; Bazellières et al., 2012; Hu et al., 2012), and may serve as a mechanism that promotes the entry of microtubules into filopodia. Their localization in the proximal filopodium is consistent with this notion as this is the site where microtubule–actin filament interactions would be initiated. Indeed, septin 7 promotes the targeting of microtubules into axonal filopodia during the branching of sensory axons (Hu et al., 2012). Tropomodulin 1, an actin-filament-binding protein, also localizes to the proximal segment of filopodial actin filament bundles (Fath et al., 2011). Tropomodulins cap the pointed ends of actin filaments and inhibit their depolymerization. The localization of tropomodulins in the proximal filopodium is thus consistent with the polarity of filaments in filopodia, and may serve to stabilize the cytoplasmic ends of filopodial actin filaments.

The filopodial shaft, including the proximal segment, contains a variety of molecules with relatively homogeneous or heterogeneous distributions. A major role of filopodia is to act as a sensory structure to detect extracellular signals. Expectedly, receptors for a variety of cell adhesion, extracellular molecules and growth factors target to filopodia. As an example, immunolocalization of the p75 neurotrophin receptor reveals that it is rather evenly distributed throughout filopodia, while the TrkA neurotrophin receptor exhibits a more punctate distribution (Gallo et al., 1997). However, in the case of membrane surface proteins that undergo extensive membrane traffic, little is known about the relative distributions

of membranous and cytoplasmic pools in filopodia. The filopodial shaft also contains signaling molecules (e.g. focal adhesion kinase (FAK), Rac1/Cdc42/RhoA; Renaudin et al., 1999; Thies and Davenport, 2003; Myers et al., in press), most of which are known to regulate the actin cytoskeleton and/or substratum attachment. The filopodial shaft exhibits multiple substratum attachment points (Steketee and Tosney, 2002). Interestingly, inhibition of substratum attachment along filopodia promotes the advance of adjacent lamellipodia along the shaft of the filopodium (Steketee and Tosney, 2002), indicating that filopodial shaft adhesions negatively regulate lamellipodial advance. A major regulator of actin filament turnover, actin depolymerizing factor/cofilin, also targets to growth cone filopodia (Gehler et al., 2004). Myosin motor proteins are found in filopodia, and these may contribute to intrafilopodial traffic (myosin I, V, X; Lewis and Bridgman, 1996; Evans et al., 1997; Kerber et al., 2009) and contractility (myosin IIA, IIB; Rochlin et al., 1995; Fig. 3.3C). Filopodia have also been reported to contain MAP1B, a microtubule-associated protein, which, however, can also bind actin filaments (Bouquet et al., 2004). MAP1B in filopodia may assist in the interactions between actin filaments and microtubules during growth cone guidance and process branching. Filopodia are sites of active membrane turnover (Section 3.3), and contain vesicle and associated proteins with known functions in synaptic vesicle biology (e.g. synaptotagmin; Kraszewski et al., 1995; Greif et al., in press) and neurotransmitter recycling systems (e.g. dopamine transporter, vesicular glutamate transporter; Rao et al., 2012; C. Cheng et al., 2002). Ezrin/Radixin/Moesin (ERM) proteins also target to filopodia (Ramesh, 2004; Gallo, 2008; Antoine-Bertrand et al., 2011; Marsick et al., 2012). ERM proteins can mediate the attachment of the membrane to actin filaments and are regulated by phosphorylation, which activates the proteins. Phosphorylated forms of ERM proteins preferentially target to filopodial shafts and positively contribute to the increase in the levels of filopodial β1-integrin and L1 induced by nerve growth factor in sensory axon growth cones (Marsick et al., 2012). Conversely, ERM proteins are dephosphorylated in response to signals that cause growth cone collapse and loss of filopodia (Schlatter et al., 2008; Gallo, 2008; Marsick et al., 2012). Finally, the shaft of filopodia can give rise to both lamellipodia and filopodia (Fig. 3.1D and Fig. 3.2A). The mechanisms regulating the initiation of protrusion along the filopodial shaft are not well understood. However, activation of phosphoinositide 3-kinase promotes the initiation of filopodia both from the main axon shaft and from the shaft of individual filopodia (Ketschek and

Gallo, 2010). In both cases, the emergence of the new filopodium is preceded by an accumulation of actin at the site of formation, as revealed by live imaging of fluorescently tagged actin. While the biological significance of the formation of new filopodia from a preexisiting filopodial shaft is not clear, these observations demonstrate that the filopodial shaft is capable of giving rise to additional protrusive activity, and thus, must contain the relevant molecular mechanisms.

The tip of the filopodium represents a specialized subfilopodial domain and atomic force microscopy measurements indicate that tips are thicker than the shaft (Grzywa et al., 2006). The tips of filopodia represent a domain where the barbed ends of actin filaments are linked to the membrane, and likely membrane spanning proteins (Faix et al., 2009). Molecules that either have been described to be specifically targeted to tips or have been shown to strongly target to tips are discussed. A recent study investigating the dynamics of the membrane spanning dopamine transporter (DAT) revealed accumulation of the levels of surface DAT at the tips of filopodia (Rao et al., 2012). The localization of surface DAT at the tips of axonal filopodia is consistent with the notion that neuronal filopodia are specialized to be precursors to presynaptic structures (discussed in Section 2.4 and further in Section 3.3). Fluorescence recovery from photobleaching experiments revealed that surface DAT at the filopodial tip exhibits low surface mobility, suggesting the existence of a mechanism that retains the DAT at the filopodial tip. The filopodial tip is generally considered to be the site of actin filament barbed end polymerization that drives filopodial extension. Consistent with this notion, Ena/VASP target to the tips of growth cone filopodia (Lebrand et al., 2004). Similarly, the actin nucleation/polymerization formins localize to the tips, and shafts, of growth cone filopodia (Matusek et al., 2008; Gonçalves-Pimentel et al., 2011). F-BAR proteins are involved in membrane deformation and contribute to the formation of filopodia (Heath and Insall, 2008). The F-BAR protein CIP4 has been detected to accumulate at the tips of filopodia and the lamellipodial leading edge during neuritogenesis (Saengsawang et al., 2012).

Although the filopodium exhibits a striking degree of subcellular organization, much remains to be learned about the mechanisms that establish this organization. It will be of interest in future studies to further detail the time course of the recruitment of the complement of proteins that target to filopodia and their subdomains, and how the dynamics of these proteins are regulated during the different phases of filopodial initiation, extension and retraction, and by extracellular signals.

3.3. Intrafilopodial Traffic

As discussed in Section 2.4, mature chemical synapses are sites of active membrane turnover. On the presynaptic side, vesicles fuse with the membrane and are endocytosed and recycled during the release of neurotransmitter into the synaptic cleft. Similarly, neurotransmitter receptors on the postsynaptic membrane undergo controlled insertion into the membrane and recycling. Recent work has identified that membrane traffic and turnover also occurs within dendritic, axonal and growth cone filopodia. Importantly, molecules associated with the cytoplasmic side of vesicles also contribute to the dynamics of filopodia.

Initial cues that growth cones secrete neurotransmitters, and may have active exocytotic mechanisms similar to those of mature presynaptic sites, were obtained from studies of developing motoneurons in vitro (Young and Poo, 1983). The formation of the synapse between a motoneuron growth cone and a nonneuronal muscle cell, termed the *neuromusclar junction*, represents the best understood instance of synapse formation to date. These synapses are fundamental to the ability of the central nervous system to control patterns of skeletal muscle activity. The establishment of synaptic responses by muscle cells in response to the arrival of a motoneuron growth cone is rapid, indicating that the presynaptic machinery is operative. Neuromuscular junctions utilize acetylcholine as a neurotransmitter. In a set of elegant experiments, the release of acetylcholine was monitored from growth cones in vitro through the use of a patch of outside-out embryonic muscle membrane affixed with a gigaohm seal at the tip of a recording micropipette (Young and Poo, 1983). The membrane at the tip of the pipette thus contained properly oriented muscle membrane acetylcholine receptors and allowed the investigators to monitor the release of acetylcholine from growth cones. Placement of this electrophysiological probe within 3–50 μm of the growth cone demonstrated activation of the muscle membrane acetylcholine receptors, revealing the release of acetylcholine from the growth cone. Consistent with these studies, synaptic vesicles have been visualized undergoing bidirectional transport and membrane fusion within growth cone filopodia (Chang and De Camilli, 2001; Sabo and McAllister, 2003; Alberts et al., 2006).

The presynaptic localization of neurotransmitter vesicle release machinery is also correlated with the formation and stabilization of axonal filopodia and branches (Cohen-Cory and Lom, 2004). As an example, in vivo dual color imaging of *Xenopus* retinal ganglion cell axons undergoing branching

in the optic tectum has revealed that axonal filopodia and branches arise from sites of the axon that contain accumulation of the synaptic vesicle protein synaptobrevin II (Alsina et al., 2001). These observations indicate that sites along the axon which contain presynaptic machinery may be specialized, or preferred, for branch addition. Consistent with this notion, molecules associated with presynaptic vesicle biology can promote the formation of filopodia. Overexpression of synaptotagmin I in nonneuronal cells, which do not normally express it, induces formation of prominent filopodia (Feany and Buckley, 1999), and overexpression of synaptotagmin I in primary cortical neurons also increases formation of filopodia (Johnsson and Karlsson, 2012). Similarly, overexpression and depletion of synaptotagmin I in chicken embryonic forebrain neurons increases and decreases the number of axonal filopodia and branches, respectively, and synaptotagmin I is found in close proximity to sites of axonal filopodial formation and also within filopodia (Greif et al., in press). Collectively, these studies provide evidence that the presynaptic vesicle release/recycling machinery has an active role in the regulation of axonal morphology both before and during synapse formation.

Active membrane recycling mechanism involving exo and endocytosis has also been shown to be required for aspects of growth cone guidance to both attractant and repellent signals. The guidance response of growth cones to gradients of signals or contact with cells in vitro is characterized by asymmetric regulation of the number of growth cone filopodia (McCaig, 1986, 1989; Oakley and Tosney, 1993; Zheng et al., 1996; Yuan et al., 2003; Robles et al., 2005). As a general rule, more and less filopodia are present on the side of the growth cone facing a source of attractant and repellent relative to the side facing away from the signal, respectively. Tojima et al. (2007) showed that the calcium-dependent attraction of sensory growth cones is mediated in part by asymmetric redistribution of vesicles, and that inhibition of the vesicle protein VAMP2-mediated exocytosis blocked growth cone turning. In contrast, clathrin-mediated endocytosis mediates growth cone responses to repellent guidance signals, and the direct asymmetric manipulation of exo/endocytosis across the growth cone can elicit growth cone turning (Tojima et al., 2010). The myosin X motor has also been shown to contribute to the targeting of receptors for the chemoattractant netrin into filopodia and at their tips (Zhu et al., 2007) and undergo intrafilopodial motility (Kerber et al., 2009). Similarly, the neurotrophin TrkB receptor undergoes intrafilopodial traffic and colocalizes with synaptic vesicle markers (Gomes et al., 2006). Sites of exocytosis along filopodia may be determined by the

filopodial targeting of the sec6/8 exocyst complex (Hazuka et al., 1999). While direct links between the regulation of endo/exocytosis and filopodial dynamics during growth cone guidance remain to be fully elucidated, it promises to be venue worth exploring. These studies emphasize that membrane turnover in filopodia is of functional significance during the early presynaptic stages of axon development.

3.4. Localized Protein Synthesis and Regulation of Filopodia

Neuronal synapses are highly localized subcellular structures that can operate independently of one another. Thus, the neuron must be able to control its physiology with a high degree of subcellular specificity. In contrast, while dendrites can be a few hundred micrometers in length, axons can be over a meter long. Thus, the neuron must also have mechanism that allows it to provide these processes, a long ways away from the cell body, with all the required cellular components (e.g. proteins and organelles). This requirement is met, at least in part, by axonal transport which delivers cargoes generated within the cell body to distal dendrites and axons. However, neurons also have the ability to locally synthesize proteins, both within dendrites and axons (Yoo et al., 2010; Jung et al., 2012). This endows neurons with the ability to locally regulate their proteome, a property termed *merotrophism* (Court and Alvarez, 2005). In addition, localized protein degradation also acts in concert with protein synthesis to control the local proteome (Twiss and van Minnen, 2006). Genome-wide screens of the mRNA content of developing and adult sensory axons identified over 1000 mRNA species (Gumy et al., 2011). mRNAs encoding cytoskeletal and cytoskeletal regulatory proteins are found in axons. Localized protein synthesis is required for alterations in synaptic function during learning and memory (Bramham, 2008), and also during the guidance of axons to their targets (Donnelly et al., 2010). This section reviews recent evidence that localized protein synthesis contributes to the formation and dynamics of neuronal filopodia.

β-actin was the first protein shown to undergo localized synthesis in the growth cones of developing axons (Zhang et al., 2001). While the mechanistic role of locally synthesized β-actin remains unclear (i.e. why does the neuron need to locally synthesize β-actin when at any time, only approximately 50% of it is in filamentous form), it is required for growth cone turning toward attractant signals (Jung et al., 2012). mRNAs are targeted into axons and dendrites through the association with ribonucleic protein particles (RNPs), which undergo transport and also regulate the availability of mRNAs for translation into protein. mRNAs associate with RNPs

through sequences usually localized to the 3′ untranslated regions. Zip code binding protein 1 (ZBP1) associates with β-actin mRNA and others (Donnelly et al., 2010). A role for ZBP1 in the regulation of filopodia was detected in ZBP1 knockout neurons. Interestingly, ZBP1 knockout affects the length, but not number, of growth cone filopodia (Welshhans and Bassell, 2011). Fragile X mental retardation protein (FMRP) is another RNP that has been involved in the regulation of growth cone filopodia. Knockout of FMRP results in increased numbers of growth cone filopodia (Antar et al., 2006). Interestingly, FMRP binds the mRNA to MAP1B (Antar et al., 2006), which negatively regulates axonal filopodia and branches (Bouquet et al., 2004). The induction of axonal filopodia by nerve growth factor is dependent on the axonal translation of mRNAs encoding for proteins that regulate the Arp2/3 complex (e.g. cortactin, Arp2; Spillane et al., 2012). Localized protein synthesis also regulates dendritic filopodia, which serve as precursors to the formation of postsynaptic structures termed *spines*. FMRP knockout neurons exhibit increased numbers of dendritic filopodia, and less spines (Antar et al., 2006; Dictenberg et al., 2008). In contrast, ZBP1 positively regulates the density of dendritic filopodia (Eom et al., 2003). Calcium-calmodulin dependent kinase I (CaMKI) phosphorylates and regulates the eukaryotic initiation factor eIF4GII, which in turn promotes protein synthesis. In dendrites, phosphorylation of eIFGII by CaMKI in response to activity promotes the formation of dendritic filopodia (Srivastava et al., 2012). Collectively, these studies indicate that localized translation of RNP-associated mRNAs in growth cones, axons and dendrites can regulate both the number and length of neuronal filopodia.

3.5. Filopodia as Signaling Domains

Filopodia act as sensors which allow neuronal growth cones and axons to respond to extracellular signals, and signaling initiated within a single filopodium is sufficient to elicit a full response by the growth cone (Section 2.2). Understanding the spatiotemporal aspects of signaling pathways in filopodia will provide insights into the mechanisms used by neurons to respond to extracellular signals. Calcium is a major regulator of the cytoskeleton and neuronal physiology, and growth cone filopodia represent subcellular domains of localized calcium signaling. Gomez et al. (2001) found that individual filopodia initiate calcium transients that result in the generation of more wide spread calcium signals in the growth cone. The filopodial transients stabilized filopodia, and when experimentally elicited asymmetrically across the growth cone, resulted in a redirection of axon extension

away from the side where transients were elicited. The stabilization of filopodia by calcium transients is in turn dependent of activation of the protease calpain within filopodia, resulting in net decreased tyrosine phosphorylation of filopodia proteins (Robles et al., 2003). A similar stabilizing role for filopodial calcium signals has been reported for dendritic filopodia (Heiman and Shaham, 2010). Some aspects of cyclic adenosine monophosphate (cAMP) signaling are also selectively regulated in growth cone filopodia. Han et al. (2007) found that the targeting of type II protein kinase A to filopodia is required for the regulation of growth cone turning by cAMP. Similarly, Nicol et al. (2011) described the presence of cAMP transients in growth cone filopodia, which in turn regulate filopodial calcium transients. Filopodia also respond to extracellular signals with increases in the levels of the signaling lipid phosphatidylinositol (3,4,5)-triphosphate (PIP3), generated through activation of phosphatidylinositol 3-kinases (PI3K). Elevations of PIP3 have been reported in both dendritic and axonal filopodia in response to neurotrophin signaling (Luikart et al., 2008; Ketschek and Gallo, 2010). Elevations in the activity of the Rho-family GTPase Cdc42 have also been detected in growth cone filopodia and found to correlate with active filopodial extension (Myers et al., in press). These studies indicate that filopodia serve as specialized signaling domains in the development of both axons and dendrites.

3.6. Biomechanics of Filopodia

Atomic force microscopy (AFM) is a versatile imaging method that can also be used to extract quantitative information on the mechanical and volumetric properties of cells (Müller and Dufrêne, 2011). AFM analysis of growth cone filopodia of cultured invertebrate and vertebrate neurons revealed that these filopodia are between 100–900 nm wide and 40–270 nm in height (Grzywa et al., 2006; Laishram et al., 2009; Xiong et al., 2009). As noted in Section 3.2, the tip of the filopodium is considered to be a specialized compartment for the active polymerization of actin filaments during filopodial dynamics. Interestingly, AFM analysis revealed that the tip of filopodia is 2.5× and 1.5× higher and wider, respectively, than the shaft of the filopodium (Grzywa et al., 2006). Relative to the lamellipodial structures of the growth cone peripheral domain, filopodial actin bundles are 30–50 nm higher (Xiong et al., 2009). Thus, filopodial actin bundles are physically prominent features of the growth cone cytoplasm.

The filopodial actin filament bundles also exhibit different mechanical properties than the adjacent lamellipodia. Young's modulus, also known

as the elastic modulus, is a metric reflective of the stiffness of a material. Although stiffness and the elastic modulus are not identical, for the case of unconstrained uniaxial tension or compression, it can be considered a measure of the stiffness of a material. The elastic modulus of filopodia is twice than that of the lamellipodium, which is characterized by a meshwork of actin filaments (Xiong et al., 2009). Moreover, the actin filament enriched peripheral domain, taken as a whole (lamellipodia and filopodia), exhibits greater stiffness relative to the central domain. The filopodium thus represents a relatively stiffer cellular subdomain than the rest of the growth cone.

Advancing growth cones generate pulling forces on the substratum (Lamoureux et al., 1989). Filopodia extend outward from the surface of the cell and are eventually retracted back into the cell. As discussed in Sections 4.3 and 4.4, the retraction of filopodia is partially driven by myosin II activity. Myosin II is a contractile mechanoenzyme that contributes to cellular contractility in nonmuscle cells by acting on actin filaments. The contact of a filopodium with another cell, or a noncellular object, can result in the pulling on the contacted surface by the filopodium (Kress et al., 2007). The force generated by a single growth cone filopodium undergoing contraction has been estimated to be on the order of 50–90 μdyn (Heidemann et al., 1990). Filopodia from myosin IIB knockout neurons exhibit decreased traction force (Bridgman et al., 2001), consistent with a myosin II-based contractile mechanism. Growth cone filopodial traction forces are considered to be determined by the engagement of myosin II-dependent retrograde flow with "clutches" formed by the interactions of the filopodium with the substratum (Chan and Odde, 2008). Individual filopodia from the same growth cone exhibit significant variations in the amplitude of force generation and slippage of the clutches (Chan and Odde, 2008), consistent with reported interfilopodial differences in the rate of retrograde flow (Mallavarapu and Mitchison, 1999). The pulling of filopodia may contribute to the forward advance of the growth cone. However, inhibition of myosin II (Turney and Bridgman, 2005; Ketschek et al., 2007), disruption of actin filaments (Section 2.2), or depletion of filopodia in vivo (Dwivedy et al., 2007) affects the rate of growth cone advance in a cell type and/or extracellular environment-dependent manner, and the effects are at least in part attributable to the role of myosin II in regulating the advance of microtubules in the growth cone (Ketschek et al., 2007; see Section 5). In conclusion, it seems unlikely that pulling by filopodia is an absolute requirement of growth cone advance, but it may contribute in some contexts.

Heiman and Shaham (2010) proposed a role for tension generated by filopodial dynamics in neuronal development. In this model, forces generated by filopodia following contact with postsynaptic partners are a component of the mechanism that stabilizes filopodia. As noted previously in Section 3.5, intrafilopodial calcium fluxes can stabilize filopodia. Neurons express mechanosensitive calcium channels and the activity of these channels impairs axon extension (Jacques-Fricke et al., 2006). Thus, membrane tension generated by the retraction of a filopodium that has attached to the surface of a target cell may elicit calcium fluxes, which in turn contribute to the stablization of filopodia. Calcium can control myosin II activity through myosin light chain kinase, which is found in filopodia (Kollins et al., 2009). Interestingly, inhibition of myosin II impairs synaptogenesis, and increases the proportion of dendritic filopodial protrusions relative to established postsynaptic structures (Ryu et al., 2006; Rex et al., 2010; Rubio et al., 2011). While the mechanism underlying the effects of myosin II inhibition on synaptogenesis remains to be fully elucidated, the forces generated by myosin II contractility in filopodia may contribute to this process.

4. INITIATION, ELONGATION AND RETRACTION OF NEURONAL FILOPODIA

4.1. Determination of the Sites of Filopodia Formation

As noted previously, neurons are highly polarized cells and the formation of filopodia from the neuronal cell surface is similarly polarized. Bray and Chapman (1985) provided the first detailed analysis of the sites of filopodia formation at the axonal growth cone and along the axon shaft. A major finding of this pioneering study was that filopodia formed with greatest frequency, and retracted with the least frequency, at the leading edge of the advancing growth cone, in alignment with the main axis of axon extension. The lateral sides of the growth cone exhibit decreased frequencies of filopodial formation and increased retraction, and the rate of formation of filopodia decreases greatly a few microns behind the neck of the growth cone as the axon consolidates. The decrease in protrusive activity just proximal to the growth cone proper is a defining characteristic of the process of axon consolidation, which gives rise to the axon shaft proper as the growth cone advances (previously discussed in Section 2.2). Also, as discussed in Section 3.3, the establishment of asymmetries in the number of filopodia at the growth often correlates with the redirection of axon extension by guidance signals. The mechanisms that regulate the specific site of filopodial

formation are not well understood. This section reviews current thinking of the mechanisms that determine how a micron to submicron domain of the neuronal plasma membrane becomes the site of the formation of a filopodium.

The establishment of filopodial asymmetries preceding turning have been shown in the context of gradients of BDNF and lysophosphatidic acid (LPA) mediated growth cone attraction and repulsion, respectively (Yuan et al., 2003), and also glutamate chemoattraction (Zheng et al., 1996). Interestingly, gradient application of these signals induces filopodial asymmetries without changing the mean number of growth cone filopodia, indicating a redistribution of the sites of filopodial formation at growth cones. Surprisingly, gradients of 2,3-butanedione monoxime (BDM), considered to be a nonspecific pharmacological inhibitor of myosins, also induced asymmetry in growth cone filopodia without affecting the total number of filopodia. These observations indicate that growth cones have a mechanism to redistribute the sites of filopodia formation while maintaining baseline levels of filopodia during gradient-mediated guidance.

Surprisingly, although as noted by the work of Bray and Chapman (1985) filopodia form heterogeneously along the growth cone perimeter, little is known about the mechanism that determines the sites of filopodia formation at growth cones. The focal ring filopodial initiation system described by Tosney et al. (Steketee et al., 2001) provides insights into subcellular specializations that underlie the initiation of filopodia at growth cones, but does unveil the mechanism by which these sites are determined. However, the focal rings correlate with sites of substratum adhesion (Steketee et al., 2001). At the growth cone, adhesion sites are dynamic and are referred to as point contacts (Renaudin et al., 1999). Thus, the mechanisms that control adhesion sites at growth cones may also regulate the sites of filopodial formation. Indeed, during growth cone guidance, extracellular attractants and repellents asymmetrically regulate growth cone adhesions sites (Myers and Gomez, 2011) and filopodial distribution (Section 3.3). Microtubule dynamics control a variety of aspects of the establishment of cell polarity and migration, including substratum attachment sites (Kaverina and Straube, 2011). Similar to the distribution of sites of filopodia formation, in growth cones, the + tips of microtubules are usually aligned with the main axis of axon extension. Microtubule dynamics may therefore contribute, at least in part, to the determination of sites of filopodia formation.

The determination of the site of formation of filopodia along axons and dendrites is somewhat better understood. While the growth cone exhibits

a striking enrichment of actin filaments, the axon and dendrites contain much lower amounts of actin filaments. Along axons and dendrites, filopodia emerge from dynamic accumulations of actin filaments both in vitro and in vivo (Andersen et al., 2005; Loudon et al., 2006; Mingorance-Le Meur and O'Connor, 2009; Ketschek and Gallo, 2010; Korobova and Svitkina, 2008, 2010; Spillane et al., 2011; Andersen et al., 2011; Spillane et al., 2012), which will be referred to as actin patches (Fig. 3.4A,B). The formation of actin patches is dependent on the Arp2/3 complex, and actin patches exhibit actin filaments consistent with Arp2/3-mediated nucleation (Korobova and Svitkina, 2008; Spillane et al., 2011). The emergence of filopodia from actin patches requires the actin-filament-binding septin 6 (Hu et al., 2012). Through in vitro reconstitution assays, septin 6 was found to target to sites of Arp2/3-medited filament branches along actin filaments (Hu et al., 2012). In axons, septin 6 in actin patches promotes the recruitment of cortactin, a stabilizer of Arp2/3-nucleated filament branches. Cortactin, in turn, promotes the formation of filopodia from actin patches (Mingorance-Le Meur and O'Connor, 2009; Spillane et al., 2012).

A similar "actin accumulation as precursors to filopodia formation" phenomenology was also reported in the growth cones of sympathetic neurons and PC12 cells (Goldberg et al., 2000), in which case structures that may be analogous to axonal actin patches were termed *splotches*. In these experiments, growth cones were deprived of nerve growth factor and were largely immotile containing minimal levels of actin filaments. Treatment with nerve growth factor rapidly induced filopodia formation, which was preceded by the emergence of splotches. Splotches, like axonal actin patches, contained Arp2/3 subunits. In our own work imaging eYFP-tagged β-actin dynamics using chicken sensory neurons cultured in nerve growth factor, in which case, the growth cones are highly motile, we have also observed instances of filopodia formation being preceded by the formation of actin filament splotches/patches (Fig. 3.4C). In this example, by 6 s, an actin patch/splotch has formed (arrowhead) and by 18 s, a filopodium has emerged (arrow). A patch/splotch to filopodium emergence sequence was most readily observed in growth cones that did not have prominent lamellipodia. In growth cones with lamellipodia, the levels of actin filaments were usually quite high, possibly obfuscating formation of splotches/patches. Rac1 is a GTPase that contributes to the formation of lamellipodial actin networks, and inhibition of Rac1 similarly decreased the number of filopodia at the growth cone and along the shaft in sensory neurons (Spillane et al., 2012). Thus, there may be conceptual similarities between the mechanism that

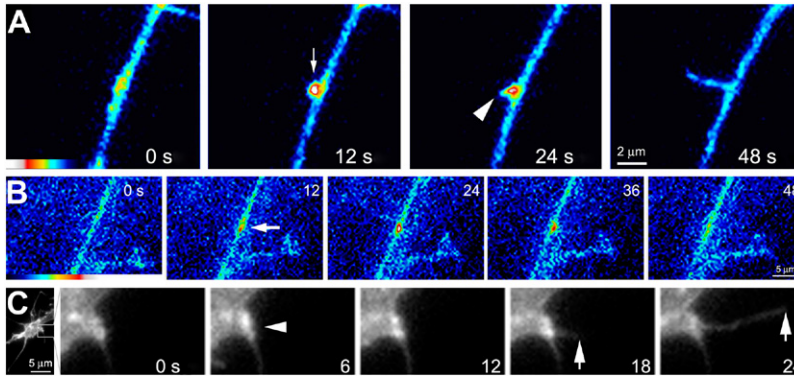

Figure 3.4 Filopodia emerge from actin precursors structures (actin patches). (A) Example of the emergence of an axonal filopodium from an actin patch precursors. The false colored timelapse shows the dynamics of eYFP-β-actin in the axon of a cultured sensory neuron. By 12 s, accumulation of actin is evident (arrow) in a patch along the axon, and at 24 s, a filopodium begins to emerge from the patch (arrowhead). By 48 s, the filopodium has extended further and the patch largely dissipated. (B) Imaging of eYFP-β-actin along sensory axons in the living acutely explanted spinal cord of a chicken embryo. Sensory neurons were transfected in vivo through in ovo electroporation (as in Spillane et al., 2011). As shown in (A), an actin patch forms at 12 s and persists until approximately 48 s. (C) Example of the emergence of a filopodium by a precursor accumulation of actin at the growth cone of a neuron expressing eYFP-β-actin. The growth cone, and the area focused upon in the subsequent panels, is shown in the leftmost panel. An accumulation of actin is evident by 6 s (arrowhead), and a filopodium emerges and extends between 18 and 24 s (arrows). For color version of this figure, the reader is referred to the online version of this book.

drives formation of filopodia at the growth cone and along the axon shaft (i.e. both emerge from precursor actin-filament-based structures), but as noted in Section 3.1, there are also likely mechanistic differences.

Along axons, the sites of actin patch formation are determined by localized microdomains of PIP3 signaling, which exhibit similar temporal profiles as the patches (Ketschek and Gallo, 2010). As PIP3 levels increase, so does the actin content of the patch, and conversely as PIP3 levels decrease, so does the actin content of the patch. PI3K signaling, leading to PIP3, is both required and sufficient to elicit actin patch formation and the formation of axonal filopodia. These studies, thus, indicate that sites of filopodia formation along axons arise in response to localized lipid-based signaling events. WAVE proteins are activators of the Arp2/3 complex and can bind to PIP3 (Derivery and Gautreau, 2010). Overexpression of WAVE1 promotes the formation of actin patches along sensory axons, but does not promote the emergence of filopodia from patches (Spillane et al., 2012). The

initial rise in PIP3 levels within microdomains may thus serve to recruit WAVE proteins to sites of actin patch formation, which in turn drive the activation of the Arp2/3 complex. Furthermore, myosin X binds PIP3, promotes formation of filopodia in a PIP3-binding-dependent manner (Plantard et al., 2010), and may serve to bundle actin filaments together during the initial stages of filopodia formation (Section 4.2). Thus, the initial rise in PIP3 levels in axonal microdomains may serve an organizational role in the formation of actin patch precursors to formation of filopodia.

In unpublished work, we have observed that axonal filopodia emerge after the peak of actin filament accumulation has occurred in patches (Fig. 3.5). The localized PIP3 microdomains follow almost identical dynamics as the actin patches they give rise to (Ketschek and Gallo, 2010). This observation thus suggests that the assembly of a filopodium from an actin patch perhaps does not correlate with the localized increase in PIP3 levels, but rather with the subsequent decline in PIP3 and actin levels. PIP3 is converted into phosphatidylinositol 4,5-bisphosphate (PIP2) by phosphatases, notably phosphatase and tensin homolog (PTEN; Park et al., 2010). Emergence of filopodia from actin patches may thus correlate with localized increases in PIP2 levels, as PIP3 is converted to PIP2 during the second half of the microdomain/patch lifespan. Lee et al. (2010) have suggested a model for the assembly of filopodia based on PIP2. In this model, PIP2 recruits proteins involved in organizing the filopodial actin bundle and establishing the tip complex that is thought to control the elongation of the filopodium (Section 4.2). Similarly, endogenous synaptotagmin 1, a synaptic vesicle-associated protein, promotes formation of axonal filopodia and branches (Greif et al., in press) and has been shown to bind to phospholipids (e.g. PIP2; Johnsson and Karlsson, 2012). Thus, the initial increase of PIP3 in microdomains drives the formation of actin patches, and, albeit speculative, the subsequent decrease in PIP3 levels and relative localized increase in PIP2 levels may serve to drive the reorganization of the filaments in the actin patch into a filopodial bundle and mediate aspects of membrane turnover during the emergence of the filopodium (Fig. 3.5B).

The formation of filopodia in neurons is under regulation by extracellular signals. Thus, it stands to reason that a signal that induces filopodia will likely induce filopodia in the vicinity of the receptors to which it binds. In the case of nerve growth factor-induced axonal filopodia and actin patches, patches and filopodia form in proximity to, or in direct colocalization with, clusters of the TrkA receptor on the surface of axons (Ketschek and Gallo, 2010). TrkA receptors associate with lipid rafts (Limpert et al., 2007), lipid

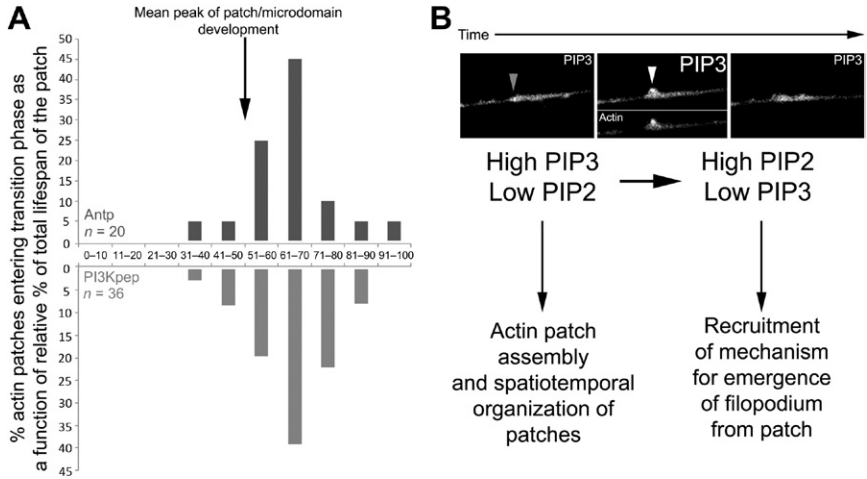

Figure 3.5 Axonal filopodia emerge from actin patches in a manner correlating with decreasing actin and PIP3 levels. (A) Actin patches and PIP3 microdomains attain their maximal intensity at approximately the midpoint of their duration (arrow; Loudon et al., 2006). Analysis of the time point during which filopodia emerge from patches, termed *the transition phase*, indicates that filopodia emerge in large part after the midpoint of patch development. The data are presented normalized to the duration of the individual patches analyzed (e.g. if the filopodium emerged at 83% of the duration of the patch, it was binned in the 81–90% bin). Activation of PI3K with a cell permeable peptide (PI3K-pep) increases the duration of patches but does not change the probability that a patch will give rise to a filopodium (Ketschek et al., 2010). The control for PI3Kpep in this data set is the cell permeable portion of the peptide (Antp; top graph). Note that although PI3Kpep increases the duration of patches, and PIP3 microdomains, it does not change the relative timing of the emergence of filopodia from patches. (B) Speculative model for the temporal regulation of axonal actin patch assembly and the emergence of filopodia from patches based on the relative levels of PIP3/PIP2 in microdomains. The images show the formation of a PIP3 microdomain (tracked using a GFP-PH domain of Akt) and an actin patch (middle panel bottom). Elevating PIP3 levels in membrane microdomains recruit molecules driving the Arp2/3-dependent formation of actin patches. The microdomain is denoted by the arrowheads in the panels, and reaches its peak intensity in the middle panel. As PIP3 levels decline following their peak, PIP2 levels increase in microdomains. The increase in PIP2 levels recruits the molecular machinery driving the emergence of a filopodium from the actin patch. Since the formation of a filopodium requires multiple molecular systems, insufficient recruitment of any required component to the microdomain may underlie the apparent stochastic nature of the emergence of filopodia from patches (6–20% of actin patches give rise to filopodia depending on developmental age of the neuron).

microdomains that have wide roles in cell signaling. A role for lipid rafts as signaling platforms underlying the formation of filopodia was shown by Scorticati et al. (2011) who report that filopodia induced by the integral membrane glycoprotein M6a require M6a targeting to lipid rafts. Thus, lipid

rafts may organize receptor systems in the neuronal membrane and contribute to the determination of the sites of filopodia formation. It is worth considering, however, that although sites of axonal filopodia formation in response to nerve growth factor codistribute with TrkA clusters, only a subset of surface membrane TrkA clusters give rise to filopodia at any given time. An additional level of spatial specificity in this system is likely attributable to the positioning of axonal mitochondria. Treatment with nerve growth factor greatly increases the colocalization of sites of actin patch and filopodia formation with axonal mitochondria, and mitochondrial respiration is required for the effects of nerve growth factor (Ketschek and Gallo, 2010). Collectively, these studies indicate that the determination of the site of formation of an axonal filopodium is likely due to the spatial convergence of multiple factors (e.g. lipid rafts, microdomains of PIP3 signaling, axonal mitochondria).

4.2. Actin Nucleation and Reorganization during the Initiation of Neuronal Filopodia

Actin filaments in neurons are very dynamic and constantly turning over. The first step in the formation of an actin filament is the nucleation of the filament from the pool of soluble actin monomers. A role for Arp2/3 in nucleating the actin patch precursors to the formation of axonal and dendritic filopodia is fairly well established (Korobova and Svitkina, 2008; Mingorance-Le Meur and O'Connor, 2009; Spillane et al., 2011). However, the role of Arp2/3 in regulating growth cone filopodia remains controversial (Strasser et al., 2004; Korobova and Svitkina, 2008; Norris et al., 2009; Gonçalves-Pimentel et al., 2011). Cordon bleu is an additional actin nucleating system that is highly expressed in the nervous system and has a role in the regulation of neuronal morphogenesis (Ahuja et al., 2007). Cordon bleu brings together actin monomers forming a seed for continued filament polymerization. Cordon bleu and Arp2/3 act independently and their contribution to actin filament levels is additive in in vitro assays. Analysis of the in vitro development of axon length and branching using hippocampal neurons revealed a contributing role for cordon bleu in the formation of axon branches but not axon length (Ahuja et al., 2007). A specific role of cordon bleu in the regulation of neuronal filopodia has not been investigated. However, as initiation of axonal filopodia is the first step in the formation of axon branches, it seems likely that cordon bleu may regulate axonal filopodia. Possibly, cordon bleu along with Arp2/3 may nucleate filaments that contribute to actin patch formation. Arp2/3 forms branched filaments from preexisting mother filaments, and cordon bleu may provide

the mother filaments. A similar scenario, where two actin nucleators act cooperatively in forming filopodia, has been reported for the DAAM formin and the Arp2/3 complex in *Drosophila* neurons (Gonçalves-Pimentel et al., 2011).

The capping of the rapidly polymerizing barbed ends of actin filaments prevents polymerization. Capping proteins have a role in the formation and dynamics of filopodia. Eps8 is a barbed end capping protein involved in the induction of axonal filopodia by BDNF (Menna et al., 2009). BDNF signaling through the MAPK pathway decreases the capping property of Eps8 and, in turn, promotes formation of axonal filopodia. Ena/VASP proteins inhibit capping of actin filament barbed ends during the extension of filopodia in fibroblasts. The EVH2 domain of Ena/VASP contains the anticapping activity, domains for binding actin and for persistent targeting to filopodial tips, and is sufficient to induce filopodia in nonneuronal cells, but not in neurons (Applewhite et al., 2007). However, netrin signaling through PKA regulates Ena/VASP-dependent formation of filopodia along axons and growth cones (Lebrand et al., 2004). These considerations suggest that in neurons, capping is regulated differently than in nonneuronal cells, and that the Ena/VASP functions mediated by the EVH2 domain may not be sufficient to elicit the formation of neuronal filopodia. The difference in these mechanisms between neurons and nonneuronal cells may reflect the biological requirement of rapid and reversible formation of filopodia by neurons in response to relevant extracellular signals (e.g. BDNF and netrin).

In contrast to a lamellipodium, or an actin patch, which contain actin filaments arranged in a range of geometries forming an interconnected meshwork (Lewis and Bridgman, 1992; Spillane et al., 2011), the mature filopodium is characterized by a parallel array of actin filaments with their barbed ends pointing toward the tip of the filopodium. How are nucleated actin filaments reorganized to give this to this architecture? Clearly, one or more mechanisms exist to bundle the filaments together during the early stages of formation of the filopodium. Ena/VASP, myosin X and fascin may all contribute to this process although the roles of myosin X and fascin in neurons are not well understood compared to Ena/VASP. The tips of actin-filament-based extension exhibit electron dense accumulations at their tips, which are generally referred to as tip complexes (DeRosier and Tilney, 2000), and actin filament bundling may occur in this subcompartment. Ena/VASP and myosin X are likely components of the tip complex. Depletion of Ena/VASP proteins blocks the formation of axonal and growth cone filopodia in response to the chemoattractant netrin (Lebrand et al., 2004)

and greatly impairs formation of filopodia in vivo (Dwivedy et al., 2007). Ena/VASP proteins cluster the barbed ends of filaments, and can protect the barbed ends from capping protein thereby allowing their polymerization (Drees and Gertler, 2008). Furthermore, Ena/VASP are found at filopodial tips throughout the elongation of the filopodium and localized underneath the membrane at the tip of the filopodium (Applewhite et al., 2007). Similarly, myosin X is involved in the formation of filopodia and targets to sites of filopodial formation (Sousa et al., 2006; Watanabe et al., 2010). One of the mechanisms through which myosin X promotes formation of filopodia is to bring actin filaments together into a bundle during the early stages of filopodial emergence (Tokuo et al., 2007). Fascin binds and bundles parallel actin filaments and it is required for formation of filopodia (Svitkina et al., 2003; Jansen et al., 2011). Fascin accumulates in emergent filopodia and may associate with the tip complex (Vignjevic et al., 2006), and cultured neurons from *Drosophila* fascin mutants exhibit greatly decreased numbers of axonal filopodia/branches (Kraft et al., 2006). Thus, any or all of these molecules may contribute to the reorganization of actin filaments during the formation of neuronal filopodia. However, it is worth considering that their functions may be redundant and perhaps engaged in a context-dependent manner (i.e. myosin X may be required for bundling of filaments during early stages of the induction of filopodia by signal X but not Y, in which case fascin may be required). Alternatively, the role and requirement of these molecules may be sequential (i.e. myosin X initiates filament bundling and fascin maintains it). Finally, CRP1 is an additional actin-bundling protein that also targets to filopodia and positively contributes to the regulation of neuronal filopodia in an actin-binding-dependent manner (Ma et al., 2011). Collectively, these studies indicate that there is likely redundancy in the molecular mechanisms that can bundle filaments during filopodia formation. It will be of interest to determine if these different bundling proteins are required at different stages of filopodial development, and further detailed additional functions of the proteins that may contribute to the formation of filopodia.

Ultimately, the complement of molecular mechanisms that control reorganization of actin filaments during the emergence of filopodia need to be understood in the context of the actual underlying ultrastructural changes. A careful electron microscopic analysis of actin filament organization in growth cone filopodia revealed that, when detectable, at the base of the filopodium, one filament has its barbed end oriented toward the base of the filopodium (Lewis and Bridgman, 1992). The presence of one or more antiparallel actin filaments in the filopodial bundles has implications for myosin

II-based contractility, which utilizes antiparallel arrays (discussed further in Section 4.2). The possible presence of antiparallel filaments in filopodia also has significance in terms of the basal ring model of filopodial formation (Steketee et al., 2002). In this model, the basal ring nucleates, or recruits, a radial array of actin filaments with their barbed end embedded within the ring, and the pointed ends extending outward toward the cell leading edge. The work by Steketee et al. (2002) also identified submembranous structures they term marginal specializations, likely reflecting of the tip complex, which were opposed to basal rings and may interact with them. In the basal ring model, the bundling of filaments in an emergent filopodium occurs following the initial formation of a "nub" from the surface of the neuron. The nub reflects the accumulation of actin filaments and other unknown constituents. It will be of interest to further investigate the molecular components of nubs, tip complexes and basal rings in order to assign them specific spatiotemporal functions in the early stages of the emergence of filopodia.

Rho-family GTPases have roles in the regulation of filopodia. Cdc42 is the GTPase that was originally considered to drive filopodia formation, as evidenced by studies in nonneuronal cells (Nobes and Hall, 1995). However, roles for additional GTPases in the regulation of filopodia have emerged and Cdc42 is not required for filopodia formation in all cells/contexts (Faix et al., 2009). In neurons, Cdc42 can regulate aspects of filopodia formation, particularly at the growth cone (Meberg and Bamburg, 2000; Spillane et al., 2012). Similarly, Rac1 is classically considered to drive formation of lamellipodial structures (Nobes and Hall, 1995), but can also contribute to filopodia formation. In *Caenorhabditis elegans*, the actin-binding protein UNC-115 mediates aspects of Rac1-dependent axon guidance and filopodial dynamics (Yang and Lundquist, 2005). In sensory neurons, Rac1 drives the formation of axonal actin patches, from which filopodia subsequently emerge, and also contributes to the regulation of growth cone filopodia (Spillane et al., 2012). Similarly, netrin increases the number of growth cone filopodia in a Rac1-dependent manner (Shekarabi et al., 2005), and activation of Rac1 regulates growth cone filopodia in *Drosophila* neurons (Matsuura et al., 2004). At the growth cone, Rac1 may be involved in generating lamellipodial substrates for the emergence of filopodia in a network convergent elongation mechanism (Svitkina et al., 2003). Inhibition of RhoA, or its downstream kinase RhoA-kinase, also promotes formation of filopodia both at growth cones and along axons (Gehler et al., 2004; Loudon et al., 2006; Chen et al., in press). Along axons, RhoA kinase regulates myosin II

activity, which in turn suppresses the emergence of filopodia from actin patch precursors (Loudon et al., 2006). A recent report found that p120 catenin is required for the formation of filopodia along the axons of cultured spinal neurons (Chen et al., in press). p120 catenin can regulate Rho-family GTPases and a truncated catenin not able to do so acts a dominant negative resulting in suppression of filopodia formation from along the axons. The effects of catenin are likely to be due to regulation of RhoA, which suppresses the formation of axonal filopodia (Chen et al., in press). Semaphorin 3A causes growth cone collapse and shuts down production of filopodia and lamellipodia in a RhoA- and RhoA-kinase-dependent manner, and also blocks the formation of axonal filopodia through a RhoA-kinase-dependent inhibition of the formation of axonal actin patches (Gallo, 2006). There is much crosstalk between these GTPases and a full story in neurons has not yet emerged. However, it seems likely that different GTPases may be able to tap into the mechanism of filopodia formation in an intra- and extracellular context-dependent manner.

4.3. Polymerization, Deploymerization and Retrograde Flow of Actin Filaments in Filopodia

Depending on the extracellular context and neuron type in vitro, the tips of neuronal filopodia extend and retract with rates in the 0.1–0.2 μm/s range (Gehler et al., 2004). In vivo filopodial dynamics are attenuated, likely due to being in a three-dimensional environment with cell-to-cell membrane interactions available on all sides of the filopodium, and having to maneuver through a cellular environment. However, in vitro and in vivo filopodia exhibit a range of behaviors characterized by bending at hinge points, giving rise to new filopodia from the shaft of existing filopodia, and lateral swinging motions of the whole filopodium (Portera-Cailliau et al., 2003; Fig. 3.1). During the life cycle of an individual growth cone filopodium, the tip can undergo alternating bouts of elongation and retraction, suggesting temporary imbalances in the regulation of the mechanisms that control tip extension and retraction. In growth cone lamellipodia, actin filaments polymerize mostly with their barbed ends directed toward the leading edge, and then subsequently undergo retrograde flow toward the central domain in a myosin II and filament polymerization-dependent manner (Lin et al., 1996). Similarly, within individual growth cone filopodia, actin polymerization and retrograde flow determine the behavior of the filopodium (Mallavarapu and Mitchison, 1999). Even within a single filopodium, the rate of polymerization and retrograde flow can be regulated during its lifecycle,

and similarly, these two parameters differ between filopodia as they engage in different behaviors. Inhibition of myosin II results in longer filopodia (Lin et al., 1996; Gehler et al., 2004), consistent with a decrease in retrograde flow allowing filopodial tips to extend further through actin polymerization. In contrast, activation of the RhoA/RhoA-kinase/myosin II pathway suppresses filopodia formation and extension (Loudon et al., 2006; Gallo, 2006), presumably in part by increasing retrograde flow. Ultimately, it will be important to determine how actin polymerization and retrograde flow are controlled in individual filopodia on a subsecond timescale. Calcium can drive myosin II activity through the calmodulin–myosin light chain kinase pathway (Takashima, 2009) and myosin light chain kinase is found in axons, growth cones and filopodia (Kollins et al., 2009). It will be of interest to determine if filopodial calcium transients (Section 3.5) may regulate the temporal pattern of filopodial retraction through myosin II activation. However, filopodial calcium transients can stabilize growth cone filopodia (Gomez et al., 2001), suggesting that they may match the rate of retrograde flow to that of actin polymerization, thus resulting in a stable filopodial tip. Alternatively, calcium transients may shut down both retrograde flow and stabilize filopodial actin filaments (Lankford and Letourneau, 1989). In contrast, experimental uncaging of calcium in individual filopodia of invertebrate growth cones promotes filopodial elongation instead of stabilization (S. Cheng et al., 2002), indicating that under some conditions, calcium transients may also promote actin polymerization and/or inhibit retrograde flow. Clearly, much remains to be elucidated with regard to the mechanisms of spatiotemporal control of filopodial extension and dynamics.

While the mechanisms that control the tip of the filopodium have received much attention, recent studies have begun to indicate that filopodial dynamics are also likely controlled by events along their shaft. ERM proteins link actin filaments to the membrane and are found in filopodia. Inhibition of ERM-mediated binding of actin filaments to the membrane increases the rate of actin retrograde flow (Marsick et al., 2012). Thus, ERM protein may act as a "clutch" linking actin filaments to the membrane and decreasing their retrograde flow (Giannone et al., 2009). Actin depolymerizing factor/cofilin sever actin filaments and promote pointed end depolymerization (Bernstein and Bamburg, 2010), and may contribute to the remodeling of filopodial actin bundles. Drebrin is a protein that binds actin filaments, and in filopodia is often found in the proximal part of the filopodium (Dun and Chilton, 2010). Drebrin controls the binding of a set of other proteins to actin filaments (Poukkula et al., 2011),

and drebrin-decorated filaments are less susceptible to the activity of actin depolymerizing factor/cofilin. Thus, drebrin binding may serve to stabilize actin filaments in the proximal filopodial shaft. In unpublished work, we have found that shRNA-mediated depletion of drebrin in sensory neurons results in an early termination of the extension of axonal filopodia. Filopodia begin to emerge from axonal actin patches, but usually do not attain lengths >1–2 μm before retracting. Thus, the absence of drebrin may destabilize actin filaments during the early phase of filopodial emergence, and drebin may normally serve to stabilize the filaments close to the base of the filopodium as its tip extends. In addition, drebrin binding to actin filaments may also negatively regulate retrograde flow by decreasing myosin II interactions with filaments, which is predicted to promote filopodial extension.

It is not currently clear what determines when a filopodial tip stops elongating. One possibility is that sufficient actin monomer delivery by diffusion fails at a certain distance from the base of the filopodium (Zhuravlev and Papoian, 2011). Alternatively, additional molecular machinery required for maintaining the tip elongating may not be effectively delivered in a timely manner beyond a certain length. It is also possible that the tip complex machinery thought to drive elongation of the filopodial tip may have a "clock," which allows the relevant signaling/biochemical mechanisms to shut down after a certain time period (e.g. if necessary proteins are post-translationally modified as a function of time and the relevant demodifying system, e.g. a phosphatase, is not available at the filopodial tip, or vice versa). Neuronal filopodia, by virtue of their dynamics and lengths, will likely be crucial in addressing these intriguing questions.

4.4. Retraction and Stabilization of Filopodia

Filopodia are dynamic in vitro and in vivo, undergoing initiation extension and retraction. The regulated stabilization of filopodia is, however, fundamental to aspects of neuronal development. The heterogeneous stabilization of growth cone filopodia is a component of growth cone response to attractant and repellent guidance signals. In a single filopodium-mediated growth cone turning toward an attractant signal (e.g. neurotrophins bound to beads or contact with the surface of a guidepost cell; see Section 2.2), the stabilization of the contacting filopodium is an early event in turning. In these cases, the stabilization of the filopodium correlates with the accumulation of actin filaments within the filopodium and the entry/capture of microtubules (Sabry et al., 1991; O'Connor and Bentley, 1993; Bentley and O'Connor, 1994; see Section 5.2). In contrast, filopodia stabilization is also involved in

growth cone turning away from repellent signals following filopodial contact. During development, extending motoneuron growth cones migrate through sclerotomal tissue and avoid the posterior sclerotome. Motoneuron growth cones making filopodial contact with posterior sclerotome cells in vitro turn away from the site of contact (Oakley and Tosney, 1993). Filopodia that contact posterior sclerotome cells do not undergo retraction and are stabilized following contact. However, the filopodial contact impairs the ability of growth cone lamellipodia to advance in the direction of contact. This establishes an asymmetry in lamellipodial protrusion at the growth cone, with more lamellipodia extending away from the site of filopodial contact, and thus contributing to redirecting the growth cone away from the contact. Inhibition of lamellipodial advance along filopodia was also noted when filopodia were experimentally stabilized through the induction of intrafilopodial calcium transients (Section 3.5). Thus, the stabilization of a growth cone filopodium, in and of itself, is not a predictor of the response of a growth cone to a guidance signal, and filopodia may be stabilized by multiple mechanisms having opposite functional outcomes.

In the context of the stabilization of the filopodium during turning toward an attractant, the stabilization of the filopodium may be an indirect result of the entry of microtubules into the filopodium. However, inhibition of microtubule dynamics, and thus, the entry of microtubules in filopodia during growth cone guidance induced by contact with nerve growth factor-coated beads does not prevent filopodial stabilization, although it prevents turning (Gallo and Letourneau, 2000). Conversely, treatment with the kinase inhibitor KT5926 prevents growth cone turning toward nerve growth factor-coated beads, but does not prevent the stabilization of the contacting filopodium, indicating additional KT5926-sensitive mechanisms are operative, and filopodial stabilization by itself is not sufficient for turning (Gallo et al., 1997).

The cytoskeletal proteins that regulate the stabilization of filopodia during guidance are not well understood. Gelsolin is an actin filament severing protein that is found throughout axons and growth cones (Lu et al., 1997). Surprisingly, neurons from the gelsolin knockout mouse exhibit no obvious morphological abnormalities. However, a careful morphometric screen revealed that gelsolin knockout neurons exhibit an increased number of filopodia along their axon, and that this is due to retention of filopodia that would normally retract (Lu et al., 1997). The tip of filopodia of gelsolin knockout neurons extended at the same rate as wild-type neurons, but exhibited decreased rates of retraction and increased time spent pausing.

These data suggest that localized regulation of gelsolin activity in filopodia may contribute to the stabilization of filopodia during responses to guidance signals. Actin depolymerizing factor and cofilin, similar to gelsolin, also sever actin filaments. Overexpression of these molecules resulted in an increased number of filopodia at growth cones, but a decreased number of filopodia along the distal axon (Meberg and Bamburg, 2000). Although this study did not address the dynamics of filopodia, it suggests that actin depolymerizing factor and cofilin may also have a role in regulating filopodial stability, and if so, differently at the growth cone and along the axon.

5. FILOPODIA: LINKING THE ACTIN AND MICROTUBULE CYTOSKELETON

5.1. Concerted Dynamics and Organization of the Neuronal Microtubule and Actin Cytoskeleton

Although there is no molecular similarity between actin filaments and microtubules, and the two cytoskeletal systems do not interact directly, it has become clear that indirect mechanisms exist in neurons linking the two systems both at the mechanical and molecular level. Original insights indicating that microtubules could regulate aspects of actin cytoskeletal dynamics came from studies of the effects of microtubule disrupting agents on the motility and dynamics of nonneuronal cells (Bershadsky et al., 1991; Kaverina and Straube, 2011). Similarly, attenuating microtubule tip dynamics, depolymerizing microtubules or causing aberrant microtubule polymerization have all been shown to affect actin-filament-based protrusion in neurons (Bray et al., 1978; Tanaka and Kirschner, 1991; Gallo, 1998). Conversely, actin filaments and myosin II activity regulate the distribution of microtubule tips in growth cones (Schaefer et al., 2002; Burnette et al., 2007; Ketschek et al., 2007). Recent advances have begun to shed light on the molecular mechanisms that link microtubules and actin filaments. The following sections address how the actin and microtubule cytoskeleton is integrated during growth cone guidance mediated by filopodia and axon collateral branching.

5.2. Actin and Microtubules in Filopodia-Mediated Growth Cone Guidance

Growth cone guidance in response to extracellular signals requires both actin filaments and microtubules. Actin-filament-based structures (filopodia and/or lamellipodia) are generally considered to be required for the

extending growth cone to sample its environment and respond appropriately. The response of a single filopodium to a positive/attractant guidance signal is sufficient to reorient the growth cone. However, for guidance to occur, the filopodium must be invaded by one or more microtubules (Gallo and Letourneau, 2000; Dent and Gertler, 2003), which provide structural support for the development of a new segment of axon shaft in the direction of the signal. Microtubules will also allow for the organelles in the central domain of the growth cone to undergo vectorial redistribution toward the signal (Lin and Forscher, 1993).

Microtubules can penetrate individual growth cone filopodia (Fig. 3.6, arrowheads), and similarly, axonal filopodia (Fig. 3.6, arrow). When axons are undergoing extension in the absence of guidance signals, the penetration of microtubules in filopodia is likely randomly distributed along the growth cone perimeter. However, individual filopodia that make contact with a source of a guidance cue (e.g. the surface of a cell) become invaded by one or more microtubules and retain the microtubule(s) while the growth cone reorients. Thus, there may be two major regulatory mechanisms in this process: (i) regulation of the entry of microtubules in filopodia and (ii) the retention/stabilization of microtubules in the filopodia. Both these mechanisms likely impact the dynamic instability of microtubule tips, which otherwise exhibit cycles of polymerization and depolymerization occurring on a much smaller timescale (a few tens of seconds) than the reorientation of the growth cone (minutes to tens of minutes).

Molecules that link the actin and microtubule cytoskeleton, physically or functionally, have been identified and suggested to have roles in growth cone guidance (Vitriol and Zheng, 2012). However, direct analysis of the

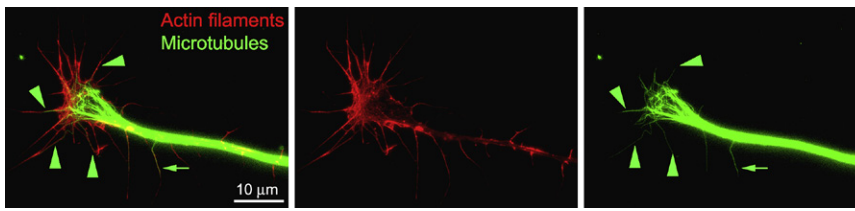

Figure 3.6 Microtubules enter neuronal filopodia. Example of a cultured sensory axon stained to reveal actin filaments (red) and microtubules (green) following combined fixation and extraction, a protocol which removes soluble tubulin but retains tubulin in polymeric form (i.e. microtubules) (Gallo and Letourneau, 1999). Microtubules are found in subsets of filopodia both at the growth cone (arrowheads) and along the axon shaft (arrow). For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

roles of these proteins, or signaling mechanisms, in the cytoskeletal dynamics underlying filopodia-mediated growth cone guidance is minimal. Surprisingly, very little is known about how microtubule tips are targeted and retained in filopodia. Thus, understanding the mechanisms that regulate the targeting or retention of microtubules in filopodia during guidance remains a frontier. Even less is known about whether microtubules are negatively regulated in growth cones during guidance in response to repellent signals.

Bentley et al. established the fundamental sequence of events underlying growth cone turning toward attractants through single filopodial contact (Sabry et al., 1991; O'Connor and Bentley, 1993; Bentley and O'Connor, 1994). In a series of papers, they used live imaging approaches to describe the cytoskeletal (actin and microtubule) reorganization underlying the guidance of grasshopper Ti1 pioneer growth cones in the developing limb bud. This guidance event is initiated and mediated as a filopodial contact with specific guidepost cells. In filopodia that contact targets, actin filaments initially accumulate at the base of the filopodium and also undergo a degree of retrograde flow toward the body of the growth cone. In concert with the ensuring alterations in the filopodial actin cytoskeleton, microtubules were observed to undergo excursions into and out of filopodia, as well as retention in filopodia that demarcated the future direction of growth cone extension. A similar sequence of cytoskeletal reorganization has been described in the growth cones of sensory neurons undergoing guidance toward a source of nerve growth factor (Gallo and Letourneau, 2000).

Using the growth cones of *Aplysia* bag cell neurons, Forscher et al. have provided additional insights into the interactions between actin filaments in filopodia and growth cone microtubules during guidance, again through live imaging approaches. In these growth cones, microtubules often align with filopodial actin filament bundles. Microtubules align and polymerize along filopodial actin bundles, but also engage actin retrograde flow and are in turn cleared from the peripheral domain (Schaefer et al., 2002). Specific depletion of filopodial actin bundles resulted in increased penetration of microtubule tips in the growth cone peripheral domain, and decreased retrograde flow of microtubules toward the central domain (Burnette et al., 2007). Actin filament retrograde flow is in part mediated by myosin II (Lin et al., 1996). Consistently, inhibition of myosin II promotes the distance that microtubules penetrate filopodia (Ketschek et al., 2007). Finally, myosin II is required for growth cone turning at substratum borders and in response to nerve growth factor (Turney and Bridgman, 2005; Loudon et al., 2006), although the failure to undergo guidance may also be related to a more

general role of myosin II in maintaining the growth cone polarized. As discussed further in Section 5.3, septin 7 localizes at the base of axonal filopodia and promotes the localization of microtubules into filopodia, and septin 7 binds both microtubules and actin filaments (Hu et al., 2012). Interestingly, septin 7 is also found at the base of growth cone filopodia suggesting that it may have a similar role in microtubule capture during guidance (Fig. 3.7). Collectively, these studies suggest that localized regulation of myosin II during growth cone guidance could serve as a mechanism for controlling the invasion of filopodia by microtubules, which may be captured by septins.

Microtubule motors may also have a role in regulating the interactions between the actin and microtubule cytoskeleton during growth cone-mediated guidance. The microtubule motor dynein may act opposite to myosin II-based retrograde flow, thus allowing microtubules to enter filopodia (Myers et al., 2006). Kinesin 12 regulates aspects of the interactions between microtubules and filopodial actin bundles (Liu et al., 2010). Similarly, kinesin 5 negatively regulates the advance of microtubule tips in growth cone filopodia and contributes to the establishment of asymmetries in microtubules across the growth cone when turning toward beads coated with nerve growth factor (Nadar et al., 2008). Growth cones also turn toward the source of a pharmacological inhibitor of kinesin 5

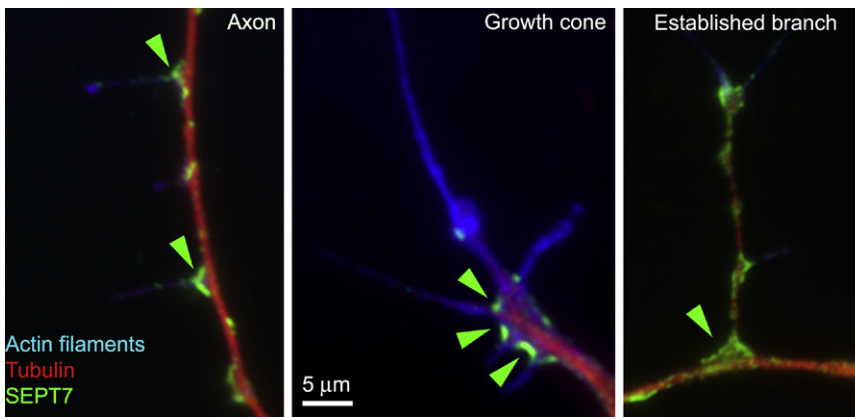

Figure 3.7 Septin 7 (SEPT7) immunolocalization in sensory axons. SEPT7 targets to the base of filopodia both along the axon shaft (left panel) and in growth cones (middle panel). Accumulations of SEPT7 at the lateral sides of the base of filopodia are denoted by green arrowheads. SEPT7 is also found at the base of established collateral branches (right panel), indicating that it is retained there after the filopodium has matured into a branch. These data were obtained through collaborative work with Dr E. Spiliotis (Drexel University) (Hu et al., 2012). For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

(Nadar et al., 2012), although a specific role for microtubule invasion of filopodia was not addressed.

The identification of a molecule as required for guidance does not, in and of itself, shed light on the underlying mechanism, it merely involves it in guidance. Ultimately, it will be required to understand where and when specific molecules act in the growth cone to coordinate the sequence of localized signaling and cytoskeletal events that result in the coupling of the actin- and microtubule-based cytoskeleton. Due to technical reasons, many studies rely on inferences from the effects of depleting or overexpressing a molecule of interest in neurons over prolonged time periods. The interpretation of these types of experiments is clearly subject to many caveats as molecules often have multiple roles in different domains of the neuron. For example, chronic depletion of a motor protein is likely to have cell-wide effects, and affect multiple cellular systems, which could in turn generate nonrelevant effects when assayed in subcellular compartments such as filopodia. Experiments designed to directly address the issues of guidance will require high spatiotemporal resolution imaging studies to determine how cytoskeletal dynamics are affected during guidance under conditions where the molecule of interest is acutely inhibited or activated (Nadar et al., 2012).

5.3. Actin and Microtubules in the Formation of Axon Collateral Branches

The entry and capture of a microtubule into an axonal filopodium are required events in the maturation of a collateral branch (Section 2.3; Gallo, 2011). The formation of axon collateral branches provides an excellent model system for studying interactions between the actin and microtubule cytoskeleton. Branches arise from specific sites along the axon and thus represent a highly spatially restricted cellular process. Furthermore, in axons, microtubules are aligned in parallel arrays and their tips tend to polymerize in a most linear manner toward the distal axon. In contrast, the base of most axonal filopodia is oriented orthogonal to the direction of microtubule tip polymerization. Interestingly, the mechanisms of axon branching and growth cone guidance have been suggested to be similar by multiple reviews (Dent and Gertler, 2003; Schmidt and Rathjen, 2010; Gallo, 2011).

As noted in Section 3.2, the base of the filopodium is the site where microtubules and the actin filament bundle of axonal filopodia first come into contact. Two proteins that interact with both actin filaments and microtubules, or associated proteins, specifically target to the base or proximal

segment of filopodia; septin 7 and drebrin. Drebrin binds to EB3 (Geraldo et al., 2008; Bazellières et al., 2012), a protein that specifically associates with the actively polymerizing tips of microtubules (Galjart, 2010). Overexpression and depletion of drebrin increase and decrease, respectively, the number of axonal filopodia and branches in vitro and in vivo (Dun et al., 2012; A. Ketschek and G. Gallo, data not shown). While it is not yet clear whether the association of drebrin with EB3 may have a functional role in targeting microtubules into axonal filopodia, this mechanism is worth considering in future studies aimed at detailing the mechanistic aspects of drebrin function in axons. Septin 7 is required for axon collateral branch formation (Hu et al., 2012). Furthermore, septin 7 was found to bind both purified actin filaments and microtubules. Neither overexpression nor depletion of septin 7 affected the formation of axonal filopodia, suggesting that its actin filament binding does not functionally regulate actin dynamics during the formation of filopodia, but may serve to target it to filopodia. In contrast, septin 7 overexpression and depletion increased and decreased the percentage of axonal filopodia that contained microtubules, respectively, indicating that septin 7 is involved in either the targeting or the retention of microtubules in filopodia during the early stages of branch formation. Since both drebrin and septin 7 localize to the proximal portions of filopodia, they may have complementary or individual roles in the regulation of microtubules in filopodia. Septin 7 is found at the base of filopodia while drebrin extends further into the proximal filopodial shaft. Thus, septin 7 may act to promote microtubule entry into filopodia, while drebrin may promote their retention in filopodia, or assist in guiding the polymerizing EB3-decorated microtubule tip further into the filopodium. Interestingly, septin 7 is retained at the base of established branches (Fig. 3.7), suggesting it may have a continued role in targeting or maintenance of microtubules in branches.

Similar to the regulation of microtubule targeting into growth cone filopodia, kinesins and microtubule-associated proteins have also been involved in the formation and elongation of axon branches. Depletion of kinesin-12 decreases the number of axonal filopodia and collateral branches (Liu et al., 2010), through unknown mechanisms. However, kinesin-12 was found to associate with actin, suggesting it may link microtubules to actin or perhaps regulate actin dynamics in an unexpected manner. In contrast, knockout of the kinesin KIF2A does not alter the number of axon collateral branches but results in longer branches (Homma et al., 2003).

The role of KIF2A in the regulation of collateral length is to negatively regulate microtubule polymerization through its depolymerizing activity. KIF2A may thus work in concert with other molecular systems in filopodia (e.g. drebrin and septin 7) to promote the maintenance and continued polymerization of microtubules in axonal filopodia, thereby promoting branch elongation. Finally, the actin and microtubule-binding protein, MAP1B, negatively regulates the number of axonal filopodia and branches (Bouquet et al., 2004), albeit through unknown mechanisms.

6. CONCLUDING REMARKS

The study of filopodia in highly polarized neurons has provided multiple insights into the complexity of an otherwise rather simple structure in appearance, the filopodium. Some of the aspects of neuronal filopodia may be restricted to neurons due to this cell type's complicated biology. However, the insights gained by the study of neuronal filopodia may be considered jump off points for the continued analysis of nonneuronal filopodia beyond the canonical signaling and cytoskeletal perspectives. Aspects of neuronal structures derived from filopodia may hold cues for the understanding of analogous nonneuronal structures. For example, the invadopodia of breast cancer cells exhibit many similarities to axon collateral branches spanning both the signaling mechanisms (e.g. PI3K) and the role of microtubules and actin cytoskeleton regulatory proteins (Murphy and Courtneidge, 2011; Yamaguchi et al., 2011). The study of neurons has also emphasized the role of localized protein synthesis in the regulation of cell morphology and physiology, and lessons learned from neurons may well apply to other cells and disease states (Shestakova et al., 1999). As we seek an understanding of the generalities underlying cellular function, the study of some of the most peculiar and specialized cells in nature is likely to reveal unappreciated, but general, complexities (DeRosier and Tilney, 2000).

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CHAPTER FOUR

Actin Isoforms in Neuronal Development and Function

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Abstract

The actin cytoskeleton contributes directly or indirectly to nearly every aspect of neuronal development and function. This diversity of functions is often attributed to actin regulatory proteins, although how the composition of the actin cytoskeleton itself may influence its function is often overlooked. In neurons, the actin cytoskeleton is composed of two distinct isoforms, β- and γ-actin. Functions for β-actin have been investigated in axon guidance, synaptogenesis, and disease. Insight from loss-of-function in vivo studies has also revealed novel roles for β-actin in select brain structures and behaviors. Conversely, very little is known regarding functions of γ-actin in neurons. The dysregulation or mutation of both β- and γ-actin has been implicated in multiple human neurological disorders, however, demonstrating the critical importance of these still poorly understood proteins. This chapter highlights what is currently known regarding potential distinct functions for β- and γ-actin in neurons as well as the significant areas that remain unexplored.

1. INTRODUCTION

While significant previous work has focused on the role of actin-binding proteins in regulating actin dynamics in neurons, one often overlooked aspect of actin biology is how the composition of the actin cytoskeleton itself influences its function. The goal of this chapter is to present what is currently known about the contribution of individual actin isoforms to the maintenance and function of the actin cytoskeleton in neuronal development and function. We will also highlight the significant areas that remain unexplored.

2. NEURONAL ACTIN CYTOSKELETON

The actin cytoskeleton contributes directly or indirectly to nearly every facet of neuronal development and function, and thus the impact of this ubiquitous protein cannot be overstated. Beginning with the birth of a neuron, an actin-based contractile ring facilitates the cellular cleavage event separating the postmitotic neuron from its neural progenitor cell (Levayer and Lecuit, 2012). Soon after, actin dynamics contribute to propelling newly generated neurons from their birthplace to destinations throughout the brain (Ayala et al., 2007). Later, axons capped by growth cones elongate in search of proper synaptic targets amidst a complex and constantly changing environment (Lowery and Van Vactor, 2009). The actin cytoskeleton not only provides a structural scaffold for the growth cone but also contributes to its guided migration facilitating the proper pathfinding of axons and assembly of neural wiring (Dent and Gertler, 2003). As neurons mature and

integrate into complex synaptic circuits, the actin cytoskeleton within dendritic spines drives the postsynaptic remodeling required for synaptic plasticity, learning, and memory (Cingolani and Goda, 2008). Throughout all these cellular events, the dynamic polymerization and remodeling of actin filaments serves to form molecular scaffolds, promote vesicular transport, and induce membrane remodeling as well as a myriad of other functions. Collectively, these actin-driven processes facilitate neuronal morphological changes that are required for the proper development and function of the nervous system. Actin dynamics have also been implicated in the regulation of gene expression, extending the reach of actin into the nucleus (Visa and Percipalle, 2010). Considering these widespread functions, it is perhaps not so surprising that the neuronal actin cytoskeleton is composed of multiple, distinct actin isoforms.

3. ACTIN ISOFORMS

The mammalian genome contains six different actin isoforms encoded by six distinct genes (Vandekerckhove and Weber, 1978). The actin isoforms were initially discovered based on their differential mobility following isoelectric focusing, which resulted in the migration of three distinct spots of actin: α-actin, the most negatively charged/acidic, followed by β-actin and the most positively charged/basic γ-actin. Within these three general isotypes, there are three α-actin isoforms named for where they are predominantly expressed including α-skeletal, α-cardiac, and α-smooth actin for skeletal, cardiac, and smooth muscles, respectively. Along with β-cytoplasmic actin, the two forms of γ-actin, γ-smooth and γ-cytoplasmic actin, round out the remaining members of the mammalian actin isoform family.

Interestingly, no actin isoform differs from another by more than 7% at the primary amino acid sequence level. The two most similar actin isoforms, β-cytoplasmic and γ-cytoplasmic actin, differ at only four out of 375 amino acids clustered at the N-terminus (Fig. 4.1). Even more striking is that the amino acid substitutions between β- and γ-cytoplasmic actin are biochemically conservative, with aspartic acid substituted for glutamic acid at positions one through three, and valine substituted for isoleucine at position 10 in γ-actin compared to β-actin. Actin isoform primary amino acid sequences are completely conserved from birds to mammals, suggesting that selective pressures have maintained these actin isoforms through 300 million of years of evolution and that they likely serve at least some nonoverlapping functions (Rubenstein, 1990).

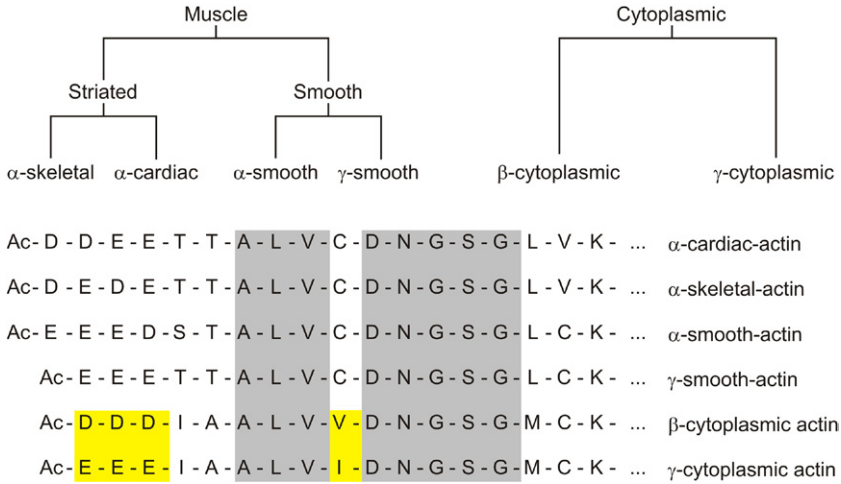


Figure 4.1 ***Higher vertebrate actin isoform family***. Actin isoforms can be split into two broad classes based on their relative expression patterns. Muscle actins are primarily found in striated (α-cardiac and α-skeletal-actin) and smooth muscle (α-smooth and γ-smooth-actin) while the cytoplasmic actins (β- and γ-cytoplasmic or simply β- and γ-actin) are found in all cells. Also shown is an alignment of the various actin isoform N-termini. Amino acids conserved across all actin isoforms are highlighted in gray while the four divergent amino acids between β- and γ-actin are highlighted in yellow. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

3.1. Expression in Neurons and the Nervous System

Clues as to what distinct roles individual actin isoforms may perform have been primarily derived from investigating potential differential expression patterns. As their name suggests, the α-actin isoforms are primarily expressed and localized to the contractile apparatus in striated and smooth muscle, while the two cytoplasmic actin isoforms, β- and γ-actin, are found in all tissues. Early work using 2D electrophoresis of proteins from whole brain and cultured neuron lysates revealed the presence of both β- and γ-actin, while no α-actin expression was detected (Choo and Bray, 1978; Flanagan and Lin, 1979). There are reports of small amounts of α-actin mRNA detected in brain preparations (McHugh et al., 1991; Tondeleir et al., 2009), but it is important to consider that this may be due to vasculature-derived α-smooth actin integrated into the brain tissue. In one study, α-cardiac actin was overexpressed in hippocampal neurons and found to form rod-like inclusions within neurites, suggesting that neurons may lack some factor important for the proper regulation of α-actin isoforms (Kaech et al., 1997). Recent work in a central nervous system (CNS)-specific β-actin

knockout (KO) mouse, however, has shown that α-smooth actin is dramatically upregulated in the brain and localized to cells morphologically resembling neurons (Cheever et al., 2012). Nevertheless, given that multiple studies have reported a predominance of β- and γ-cytoplasmic actin in roughly equal amounts within brain and isolated neuronal populations, we will focus on these isoforms, henceforth simply referred to as β- and γ-actin.

3.2. Distinct Biochemical Properties

Despite their nearly identical amino acid sequences, multiple groups have independently generated antibodies that are able to distinguish between β- and γ-actin, and revealed striking yet sometimes conflicting reports of distinct subcellular localization patterns. Early work in fibroblasts demonstrated that β-actin was often enriched at the leading edge of migrating cells, whereas γ-actin appeared to be concentrated within stress fibers and the perinuclear region (Hoock et al., 1991; Kislauskis et al., 1997; Otey et al., 1986; Shestakova et al., 2001). In addition, overexpression of β-actin in myoblasts led to an increase in cell migration and surface area, whereas overexpression of γ-actin promoted cell retraction and rounding (Schevzov et al., 1992). Collectively, these early studies suggested a specific role for β-actin in promoting cell motility at the leading edge, whereas γ-actin appeared to have a more structural or contractile role. While this apparent preferential subcellular sorting of actin isoforms is a widely held view, it is important to note that not all studies have agreed on this critical issue. Additional work with newly generated monoclonal antibodies reported essentially the opposite localization pattern, with β-actin preferentially enriched in stress fibers while γ-actin appeared to be more ubiquitously localized in multiple different cell lines (Dugina et al., 2009). Using an independent set of actin-isoform-specific antibodies verified on β- and γ-actin KO tissues and cells, our group has observed complete colocalization of β- and γ-actin in every tissue and cell type examined to date (Bunnell et al., 2011; Cheever et al., 2012; Perrin et al., 2010). Possible explanations for the discrepancies in localization patterns are discussed in detail in Section 4.1.

Recent biochemical studies from the Rubenstein group have provided additional evidence for the widely held hypothesis that β-actin is the more dynamic actin isoform and preferentially contributes to cell motility and dynamics. Using a baculovirus expression system, Bergeron et al. (2010) were able to characterize the unique biochemical properties of β- and γ-actin. In the Mg^{2+} bound form of actin generally regarded to be the most physiologically relevant, both cytoplasmic actin isoforms polymerize much

more rapidly than α-skeletal actin, but were virtually indistinguishable from each other. However, when the individual isoforms were polymerized in the Ca^{2+} bound form, β-actin exhibited significantly more rapid filament nucleation, elongation, phosphate release, and depolymerization compared to Ca^{2+}-bound γ-actin (Bergeron et al., 2010). These results collectively indicate that β-actin is the more dynamic cytoplasmic actin isoform, but essentially only in the Ca^{2+}-bound state. It was also definitively demonstrated for the first time that β- and γ-actin can readily copolymerize, with filament dynamics reflective of the actin isoform composition. Under basal conditions in most cell types where Mg^{2+} is readily available and Ca^{2+} is tightly sequestered, it can thus be expected that β- and γ-actin function essentially equivalently.

The potential for distinct dynamics of β- and γ-actin is particularly high in neurons, however, where localized Ca^{2+} transients from 1 to 100 μM can be elicited in response to chemotropic guidance cues in growth cones, at presynaptic axon terminals preceding synaptic vesicle fusion, as well as within postsynaptic dendritic spines following synaptic transmission (Augustine et al., 2003; Henley and Poo, 2004; Sabatini et al., 2002). It is perhaps no coincidence that actin, and specifically β-actin, is thought to have a crucial role in nearly all of these processes. Because actin has a higher affinity for Ca^{2+} than Mg^{2+} (Carlier et al., 1986), it is possible that these local transients could result in functionally relevant concentrations of Ca^{2+}-bound actin. Additionally, local translation of β-actin has been proposed to occur in response to chemotropic guidance cues and synaptic transmission (Leung et al., 2006; Sasaki et al., 2010; Tiruchinapalli et al., 2003; Welshhans and Bassell, 2011; Yao et al., 2006), which both involve local Ca^{2+} transients, providing another opportunity for actin to complex with Ca^{2+}. Neurons may thus represent one of the few examples where the distinct polymerization rates of β- and γ-actin may be able to influence cell development and function.

4. REGULATION OF ACTIN ISOFORMS IN NEURONAL DEVELOPMENT AND FUNCTION

Very little is known regarding the molecular regulation of actin isoforms in neurons. In the following section, we will highlight studies that have identified sometimes distinct regulation of actin isoforms predominantly in cell lines, but also with a high likelihood of relevance for neuronal development and function as well. In a few instances, specific neuronal

functions have been investigated and these findings are highlighted here and in the following sections.

4.1. Transcriptional Regulation

As introduced above, nuclear functions of actin are increasingly being uncovered with wide ranging effects on gene transcription and cellular function. Specifically, research on the transcriptional regulator serum response factor (SRF) has unveiled a potential role for actin isoforms in regulating their own and other proteins expression. SRF binds to a sequence element called the CArG box or serum response element that is present in the promoters of all actin isoform genes as well as a large number of others (Knoll and Nordheim, 2009; Miano et al., 2007). SRF is believed to be constitutively bound to these promoter elements but requires the binding of a coactivator falling into the ternary complex factor (TCF) or myocardin-related transcription factor (MRTF) families for full activity (Kalita et al., 2012). The regulation of these coactivators is thus critical for modulating SRF activity, and appears to be controlled primarily by subcellular localization of these factors as they shuttle in and out of the nucleus. The shuttling of MRTFs is particularly relevant to this discussion as it is dependent on direct binding to monomeric, globular actin or G-actin (Miralles et al., 2003). Work in fibroblast cell lines has shown that in unstimulated conditions, MRTFs are bound to G-actin, which both inhibits nuclear import and promotes nuclear export of MRTFs, thus preventing activation of SRF and transcription of target genes. When these cells are stimulated with various growth factors, actin polymerization leads to the relative depletion of G-actin as monomers are incorporated into filaments. This relieves the inhibition on MRTFs nuclear import and leads to MRTFs binding SRF and the induction of transcription (Vartiainen et al., 2007).

While much of the previous work on the SRF transcriptional pathway was worked out in cell culture and striated muscle systems, a number of recent studies have characterized neuronal and CNS roles for SRF. Ablation of SRF in the forebrain leads to impaired neuronal migration in the rostral migratory stream, corpus callosum agenesis (Alberti et al., 2005), and defects in the guidance of mossy fiber axons within the hippocampus (Knoll et al., 2006). These tissue defects correlated with impaired neurite outgrowth and axon guidance in stripe assays with primary neurons cultured from these same SRF KO mice (Knoll et al., 2006). Immunostaining and RT-PCR analysis revealed that SRF-deficient neurons had decreased levels of β-actin, raising the possibility that decreased levels of this actin isoform contributed

to the axonal phenotypes observed. However, overexpression of full length β-actin only moderately increased neurite length in cultured neurons, suggesting that deficient levels of β-actin are not exclusively responsible for the axonal phenotypes observed (Knoll et al., 2006). Beyond the development of the nervous system, additional SRF KO mouse models presented with hyperactivity (Parkitna et al., 2010) while others exhibited defects in learning and memory (Etkin et al., 2006). Given that these behavioral phenotypes are likely rooted in impaired synaptic transmission or plasticity, both of which involve actin dynamics at the pre- and post-synapse, it remains possible that the misregulation of actin isoforms may contribute at least partially to the phenotypes reported in these animals.

The CNS roles for SRF may be particularly relevant to the function of actin isoforms for two reasons. First, it was recently shown in mouse embryonic fibroblasts that ablation of β-actin but not γ-actin disrupted the polymerized or filamentous (F) actin to G-actin ratio, leading to profound changes in gene expression including some SRF targets (Bunnell and Ervasti, 2010; Bunnell et al., 2011). It was thus proposed that β-actin may specifically regulate the F- to G-actin ratio in cells, which is critical for modulating the localization of MRTFs and SRF activation. It is currently unknown but entirely plausible that β-actin may also perform a similar function in regulating the F- to G-actin ratio in neurons as well. Additionally, work in mouse models ablated for β-actin in neurons revealed a number of phenotypes including corpus callosum agenesis, hyperactivity, and memory defects (Cheever et al., 2012) that closely mimic those of SRF-deficient mouse models (see Section 6.1 for more detailed discussion). Further work will be required to definitively demonstrate that the phenotypes observed in β-actin-deficient mouse models are mediated by SRF disruption, yet the potential for modulating a large array of genes by perturbing a specific actin isoform is an intriguing idea with significant therapeutic potential.

4.2. Posttranscriptional Regulation

The posttranscriptional regulation of β-actin has been intensively studied and remains one of the most well-characterized aspects of actin isoform biology. As described above, early studies in fibroblasts reported that β-actin mRNA and protein were specifically enriched at the leading edge of motile cells, which was not observed for γ- or α-actin isoforms (Hill and Gunning, 1993; Kislauskis et al., 1993). Work primarily from the laboratory of Robert Singer described a mechanism where the specific localization of β-actin was based on a 54 nucleotide sequence in the 3′ untranslated region

(UTR) of β-actin mRNA called the *zipcode sequence* (Kislauskis et al., 1994). No other actin isoform has an analogous sequence in the 3′ UTR nor has any other actin isoform mRNA been found to be actively localized to the leading edge of a cell. At least two proteins have been shown to directly bind the zipcode sequence within the β-actin 3′ UTR, one of which, zipcode binding protein 1 (ZBP1), has been extensively characterized (Gu et al., 2002; Ross et al., 1997). ZBP1 is one member of a family of RNA-binding proteins expressed in a broad range of species (Nielsen et al., 2001; Yisraeli, 2005). ZBP1 was first cloned from chick fibroblasts and is most closely related to IMP1 in humans and mIMP or CRD-BP in mice. Less is known regarding the function of ZBP2/IMP2, while the third family member ZBP3/IMP3 is most closely related to the *Xenopus* homolog Vg1 RBP. Because the majority of work in neurons has used the ZBP nomenclature, we will use this throughout the chapter but note that ZBP family members are homologous to the mammalian IMP family.

Additional work in fibroblasts established a model whereby β-actin mRNA is bound cotranscriptionally by ZBP1, which facilitates the transport of β-actin mRNA from the nucleus to the leading edge of the cell while also inhibiting its translation to prevent ectopic synthesis (Huttelmaier et al., 2005; Oleynikov and Singer, 2003). Src kinase can phosphorylate Y396 on ZBP1, which reduces the affinity of ZBP1 for the β-actin mRNA zipcode sequence, thus freeing the mRNA for local translation (Huttelmaier et al., 2005). Treatment of fibroblasts with antisense oligonucleotides to the zipcode sequence disrupts the localization of β-actin mRNA and protein, and leads to delocalized sites of actin polymerization and reduced directional motility (Kislauskis et al., 1997; Shestakova et al., 2001). Thus, the localization and local translation of β-actin mRNA may provide one mechanism by which cells can regulate localized actin dynamics and promote directional cell migration. It is critical to note, however, that β-actin is not the only mRNA target of ZBPs. In fact, in HeLa cells, roughly 3% of the transcriptome was found within ZBP1/IMP1 ribonucleoprotein granules, including a number of RNAs with specific relevance to actin regulation in neurons including cofilin1, profilin 1, paxilin, rhoA and zyxin (Jonson et al., 2007). Thus, functions of ZBPs cannot exclusively be attributed to localization of β-actin without additional direct evidence.

Given the large excess of actin in most cells (Pollard et al., 2000), it is worth asking why cells would need to locally synthesize more actin in the first place. The simplest explanation is that locally synthesizing actin may be a mechanism to increase the G-actin concentration specifically in

localized microdomains. Because the rate of actin polymerization is directly proportional to the amount of free G-actin (Pollard and Borisy, 2003), the local synthesis of actin confined to the leading edge of a cell may be one mechanism by which actin polymerization is increased to promote cell migration. Additionally, local synthesis of actin may promote *de novo* actin filament nucleation. The cytosolic chaperone protein (CCT) is required for proper actin folding following translation, and has been shown to associate with F-actin, possibly acting as a nucleation factor itself to promote *de novo* filament formation from newly translated actin monomers (Grantham et al., 2002; Pappenberger et al., 2006). Lastly, newly synthesized actin may be more readily polymerized than preexisting actin monomers. Posttranslational modifications of preexisting actin could negatively affect polymerization, which has been demonstrated in at least one case with the glutathionylation of actin (Wang et al., 2001). Although it is unclear to what extent the population of pre-existing actin is glutathionylated, it is plausible that locally synthesized actin monomers may be rapidly integrated into filaments before glutathionylation could occur, providing an additional mechanism to promote localized actin dynamics.

4.3. Posttranslational Regulation of Actin Isoforms

In addition to glutathionylation, actin can also be acetylated (Rubenstein and Martin, 1983), arginylated (Karakozova et al., 2006), phosphorylated (Gu et al., 2003; Vandermoere et al., 2007), ubiquitinated (Burgess et al., 2004; Kudryashova et al., 2005), and SUMOylated (Hofmann et al., 2009). However, while the acetylation of muscle actins differs slightly from the cytoplasmic actins (Rubenstein, 1990), no differential posttranslational modifications of cytoplasmic actins have been identified with one intriguing exception. β-actin was reported to be specifically arginylated at its N-terminus (Karakozova et al., 2006), creating a bulky positive charge thought to be critical for promoting the branched actin networks found in lamellipodia. Indeed, fibroblasts deficient for the enzyme responsible for the arginylation of β-actin, arginyltransferase-1 (*Ate1*), exhibited abnormal morphology and impaired migration that could be rescued by a genetically encoded and permanently arginylated β-actin (Karakozova et al., 2006). *Ate1* was subsequently found to arginylate a large number of other proteins including additional sites on β-actin as well as α-cardiac-actin and a number of actin-binding proteins (Wong et al., 2007). Interestingly, arginylated γ-actin was not detected in these initial experiments. Subsequent work by this group reported that γ-actin was also arginylated, but that differences in

the nucleotide coding sequences between β- and γ-actin resulted in a more complex RNA secondary structure for γ-actin and slowing its translation rate (Zhang et al., 2010). The reduced translation speed of γ-actin is sufficient to allow a lysine residue normally rapidly buried within the actin molecule to be exposed and ubiquitinated, leading to its rapid degradation and likely explaining why arginylated γ-actin was not previously detected.

The role of arginylation and specifically the arginylation of actin isoforms is only beginning to be explored, with essentially nothing known regarding its role in neuronal development and function. A whole body *Ate1* KO mouse is embryonic lethal due to profound angiogenic remodeling and developmental heart defects (Kwon et al., 2002). However, generation of a conditional *Ate1* KO mouse through the use of a tamoxifen-inducible Cre system in adult animals did reveal important roles for arginylation and potentially the arginylation of β-actin in the nervous system (Brower and Varshavsky, 2009). In addition to being significantly smaller than control littermates, conditional adult *Ate1* KO mice had proportionally larger brains and exhibited profound hyperactivity in open-field behavioral assays. These mice also presented with seizures and an enhanced startle response suggesting heightened anxiety levels. Finally, core body temperature was also abnormal, which could have potential links to hypothalamic dysfunction. Notably, small size, potential hypothalamus defects, and profound hyperactivity are phenotypes shared with a CNS-specific β-actin KO mouse (Cheever et al., 2012), potentially linking some of these phenotypes to the specific arginylation of β-actin within neurons and the nervous system.

While the differential fates of arginylated cytoplasmic actins are likely based on nucleotide coding sequences, there is still the potential for distinct interactions between the cytoplasmic actins and actin-binding proteins, which could have significant implications for actin isoform function. Importantly, the four divergent amino acids between β- and γ-actin are exposed on the outer surface of actin filaments where they could potentially interact differentially with actin-binding proteins (Fujii et al., 2010; Oda et al., 2009). In addition, antibodies capable of distinguishing between β- and γ-actin have been generated by multiple groups (Gimona et al., 1994; Hanft et al., 2006; Otey et al., 1986; Perrin et al., 2010; Sonnemann et al., 2006), providing even more support for the possibility of proteins distinguishing the subtle differences between β- and γ-actin. Yet despite this data, there has been no definitive evidence to date supporting differential interactions of an actin-binding protein with a specific cytoplasmic actin isoform. It is worth noting however that a small number of studies have

reported stronger binding of a number of proteins including cofilin (De La Cruz, 2005), β-cap73 (Shuster et al., 1996), ezrin (Yao et al., 1996), L-plastin (Namba et al., 1992), and profilin (Larsson and Lindberg, 1988) to preparations rich in β-actin compared to muscle actin enriched samples. While intriguing, in terms of actin isoforms, this is analogous to comparing apples to oranges, and it is difficult to reconcile the significance of these findings as it pertains to differential binding of the nearly identical cytoplasmic actin isoforms.

5. ROLES FOR ACTIN ISOFORMS IN NEURONAL DEVELOPMENT AND FUNCTION

In the preceding sections, we introduced the actin isoforms and what is currently known regarding their biochemical characteristics and regulatory mechanisms. Much of this work is relatively indirect and mostly conducted with purified biochemical preparations or in nonneuronal cell types. We will review what has been learned regarding actin isoform functions in neurons from in vitro and cell culture-based studies. This encompasses the bulk of what is currently known regarding the roles of actin isoforms in neuronal development and function. These studies will then be compared to what has been learned from recently developed in vivo animal models.

5.1. Actin Isoform Expression Patterns during Neuronal Differentiation and Brain Development

5.1.1. Temporal and Protein Level Regulation

As described in the introductory sections, no endogenous α-actin isoforms have been detected in neuronal preparations, leaving only the cytoplasmic β- and γ-actins to fulfill the functions of the actin cytoskeleton in neurons. Some of the first attempts at elucidating the functions of the cytoplasmic actins in neurons assessed their expression levels and localization. Early work in PC12 neuron-like cell lines demonstrated that nerve growth factor (NGF)-induced differentiation and neurite extension resulted in a rapid increase in the expression of both β- and γ-actin mRNA, followed by a more gradual reduction in levels such that both mRNAs returned to pretreatment levels by 15 days post-NGF introduction (Henke et al., 1991). At the protein level, however, β- and γ-actin were both elevated following NGF treatment and most intriguingly, the relative composition of actin within these cells appeared to change such that γ-actin made up a significantly larger proportion of the total actin population at the end of the

treatment (Chang et al., 1986). Interestingly, we observed a larger increase in γ-actin expression compared to β-actin during hippocampal neuron differentiation in culture (unpublished results), which may also result in a similar shift in the cytoplasmic actin composition toward a lower β- to γ-actin ratio. Yet this is in contrast to similar experiments performed with whole rodent brain extracts. In one study, β-actin protein levels increased rapidly during the second half of embryonic development followed by a sharp decrease after birth. γ-actin levels increased to a much smaller percentage but did not exhibit a similar drop off, and were maintained at similar levels into adulthood (Weinberger et al., 1996).

The conflicting nature of the reports described above emphasizes the difficulty in executing informative studies examining individual actin isoform expression in rapidly changing cells. First, in some cases the reports described above were performed decades before KO tissue was available to validate the specificity of the antibodies. In addition, the cell culture experiments present the significant problem of normalization of protein levels. Neurons in culture exhibit dramatic and profound morphological changes during differentiation, with cells progressing from small spheres to extending long processes across vast distances. Thus, cytoskeletal proteins used to normalize expression studies such as these become almost useless as their levels most certainly change along with actin. In addition, commonly used alternative loading controls such as GAPDH, ribosomal subunits, or histone proteins likely also change due to the increased metabolic demands of rapidly growing and differentiating neurons. The in vivo studies with whole brain extracts can have similar normalization issues along with the added complexity of the makeup of the tissue. For example, although glial cells make up a relatively small proportion of the cells within the developing brain, their proliferation continues through late development to the point where they can substantially outnumber neurons in the mammalian adult brain. Vasculature and microglia numbers also increase during development, which may skew the actin isoform composition within the brain and complicate interpretation of data. Microglia, in particular, may be especially problematic as they have been reported to be the highest β-actin expressing cells within the brain (Plantier et al., 1998).

Direct molar comparisons between β- and γ-actin levels within neurons would be even more informative than the relative comparisons described above, but this type of experiment has only recently become even possible. While antibodies can reveal relative changes in actin isoform expression, it is not possible to directly compare levels across antibodies for β- and γ-actin.

There are two potential solutions to overcome this limitation. One would be to use a total protein stain to label isoelectrically focused samples of β- and γ-actin followed by densitometry, thus enabling direct comparisons of actin isoform levels. This experiment has several caveats, however, in that separating β- and γ-actin, which differ only minutely in isoelectric points, is inherently difficult not to mention the all too common and overlooked potential for artifactual spots. Furthermore, the actin must be purified from the total protein to enable visualization with protein stains, which is not without the potential for artifacts as well. The second method would be to generate standard curves of pure β- and γ-actin antibody staining to allow for direct determination of actual molar quantities of protein present in extracts. Until recently, there was no way to obtain biochemically relevant amounts of pure β- and γ-actin in order to generate these curves. However, with the recent report of baculovirus-mediated expression of β- and γ-actin in insect cells, this may now be possible, although contamination from endogenous insect actin remains a problem (Bergeron et al., 2010).

In summary, data from a small number of studies suggest β- and γ-actin levels likely increase during early neuronal differentiation and may be accompanied by a relative change in the actin isoform composition (Chang et al., 1986). With this shift, β-actin levels appear to decrease in total brain and neuron-like cell lysates later in development and differentiation, while γ-actin levels remain steady or increase subtly (Micheva et al., 1998; Weinberger et al., 1996). As for the actual molar composition of actin isoforms during these phases of neuronal differentiation, the available data are fragmentary at best and based on isoelectric focusing. Lysates from chick sympathetic and sensory neurons isolated at embryonic day (E) 10 and cultured for 24–36 h to allow for neurite outgrowth showed an equivalent amount of β- and γ-actin present (Choo and Bray, 1978). Actin isolated from whole brain embryonic chicks of the same age showed a similar 1:1 ratio of β- to γ-actin (Flanagan and Lin, 1979). In lysates from adult rat brains, however, the ratio was much closer to 2:1 β- to γ-actin (Otey et al., 1987). Thus, the evidence available indicates that actin isoform levels exhibit significant fluctuations during development and potentially to different extents, eventually resulting in β- to γ-actin ratios between 1:1 and 2:1.

5.1.2. Spatial Localization

As with fibroblasts, distinct localization of actin isoforms in neurons has been difficult to pin down. In early work with cultured embryonic rat cortical neurons and antibodies generated against β- and γ-actin-specific

epitopes, Bassell et al. (1998) reported that β-actin was specifically enriched in growth cones and present at much lower levels in the developing neurite shafts. In contrast, γ-actin appeared to be uniformly distributed and was essentially indistinguishable from phalloidin-stained F-actin (Bassell et al., 1998). It is important to note, however, that quantitative data was not presented here. Because growth cones are among the most actin-rich structures within neurons, and neurites themselves contain relatively little actin, poor binding of one antibody compared to another could give a similar staining pattern. Therefore, caution must be taken in interpreting non-normalized data comparing two different antibodies and concluding that the staining represents distinct localization patterns of, in this case, β-and γ-actin.

These same antibodies have also been used to stain fixed brain sections and examine the distribution of β- and γ-actin in vivo. Here again, distinct localization patterns for β- and γ-actin were reported. In one study, γ-actin was reported to be expressed throughout cell bodies and neurites while β-actin expression became progressively restricted to dendritic spines with age based on immunogold studies (Micheva et al., 1998). A similar restriction in β-actin distribution was reported by another group where β-actin expression in medulla axons progressively decreased from embryonic stages to adults, while γ-actin staining remained strong (Weinberger et al., 1996). Collectively, these studies suggest that β-actin may be restricted to more dynamic structures such as dendritic spines as development progresses, and not present in significant amounts in relatively less dynamic structures such as axon shafts, where γ-actin staining is prominent. Thus, although β-actin levels may decrease with development, the relative composition of β- to γ-actin within tightly confined yet dynamic compartments may actually increase, potentially altering the properties of the underlying actin cytoskeleton and influencing neuronal function.

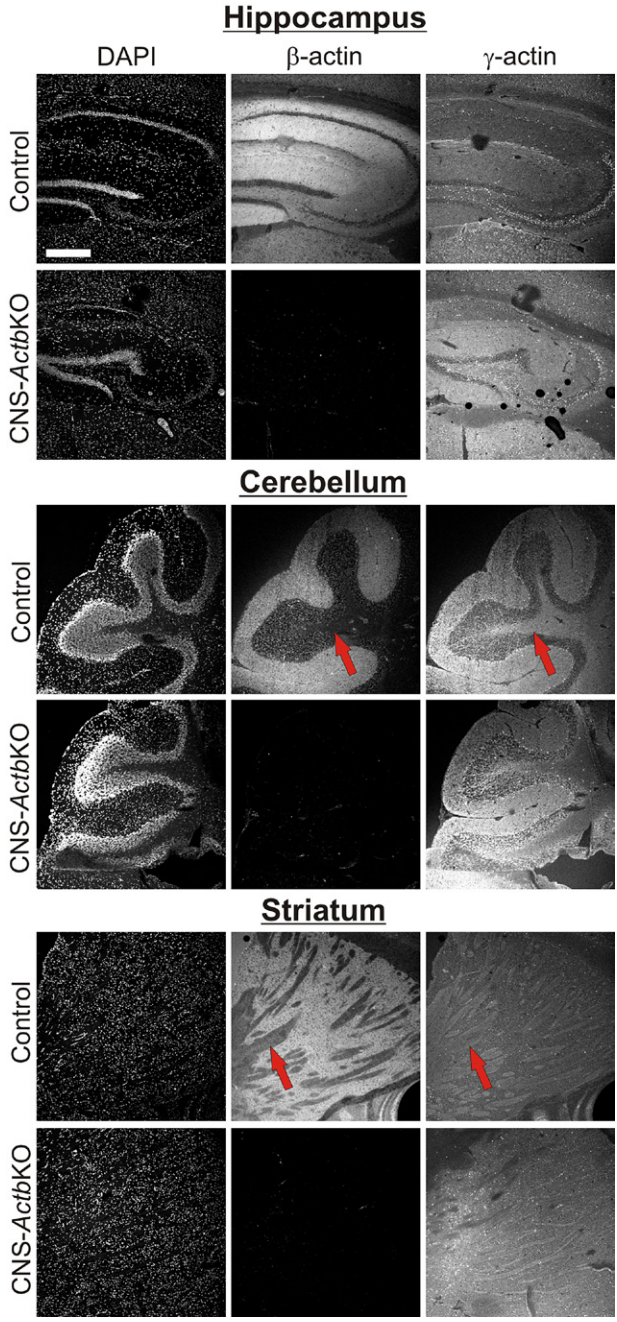
The reports discussed above place β-actin expression and localization within dynamic neuronal structures, supporting models that β-actin is the major actin isoform fueling actin dynamics. Nevertheless, some caution is warranted. First and foremost, the specificity of the antibodies used under the staining conditions described has not always been demonstrated with KO tissue. Secondly, there is now evidence that actin isoforms in some tightly packed filament actin structures may be undetectable with labeled secondary antibodies (Perrin et al., 2010). Finally, the binding of actin-isoform-specific antibodies may also be masked by formaldehyde fixation. We have found that postfixation with methanol dramatically increases staining with actin-isoform-specific antibodies, which was also recently confirmed

by two independent groups using an additional set of actin isoform antibodies (Bunnell et al., 2011; Cheever et al., 2012; Dugina et al., 2009; Perrin et al., 2010; Perycz et al., 2011). We hypothesize that brief treatment with methanol may serve to "loosen" actin-based structures, thereby unmasking binding sites for actin-isoform-specific antibodies.

Taking into account the factors just discussed, we have used primary antibodies with specificity most definitively demonstrated on β- and γ-actin KO tissue (Bunnell et al., 2011; Cheever et al., 2012; Perrin et al., 2010) to stain both dissociated neurons and brain sections to confirm the localization patterns of β- and γ-actin. Importantly, we used primary antibodies directly conjugated to fluorescent dyes and co-stained with both β- and γ-actin antibodies simultaneously in order to limit any epitope accessibility issues and allow for direct comparison of localization patterns within the same neuron or tissue section. Surprisingly, in cultured hippocampal neurons, we found β- and γ-actin to be colocalized with no evidence for preferential enrichment of β-actin at the growth cone (Cheever et al., 2012). Staining of E18 hippocampal sections also revealed no evidence for differential localization of β- and γ-actin within tissue sections (Cheever et al., 2012). We further examined the localization patterns of β- and γ-actin in three distinct regions of the adult brain (Fig. 4.2). In the hippocampus, β- and γ-actin staining was absent from nuclei but strongly localized to the stratum radiatum and stratum lacunosum moleculare, both areas rich in synapses and dendritic spines. We observed similar localization patterns for β- and γ-actin in the cerebellum as well, with both isoforms colocalized in synapse-rich regions like the molecular layer and notably absent from the cell body and nuclei-rich granule cell layer.

There was, however, one specific case where we did observe distinct localization patterns of the cytoplasmic actins. Strong staining for γ-actin

Figure 4.2 ***Localization of cytoplasmic actin isoforms in adult brains.*** Representative images from control and CNS-*Actb*KO brain sections to demonstrate antibody specificity for β-actin. Sagittal sections through the hippocampus, cerebellum, and striatum were costained with DAPI and cytoplasmic actin-specific primary antibodies directly conjugated to fluorescent dyes. In the hippocampus, cytoplasmic actin staining colocalized in synapse-dense areas including the stratum radiatum and stratum lacunosum moleculare, while staining was noticeably absent from cell bodies and nuclei. This was also true in the cerebellum with the strongest staining localized to the molecular layer. Interestingly, β-actin staining was virtually absent from the large white matter tracts in the cerebellum, whereas γ-actin was still present (arrows). A similar apparent exclusion of β-actin from large axonal tracts was also observed in the striatum (arrows). Scale bar 300 μm. For color version of this figure, the reader is referred to the online version of this book.


Hippocampus
DAPI
β-actin
γ-actin
Control
CNS-*Actb*KO
Cerebellum
Control
CNS-*Actb*KO
Striatum
Control
CNS-*Actb*KO

was found within the axonal tracts of the cerebellar white matter while β-actin staining was virtually nonexistent (Fig. 4.2, arrows). Staining of axonal tracts within the striatum revealed this same pattern, again with γ-actin present while β-actin was noticeably absent (Fig. 4.2, arrows). The lack of β-actin staining observed in large axonal bundles from adult animals is in agreement with the previous studies described above (Weinberger et al., 1996), providing further support that β-actin is either absent or at relatively low levels compared to γ-actin in large axonal bundles. However, this was the only case of distinct localization between β- and γ-actin that we observed, suggesting that β- and γ-actin may be colocalized to a much greater degree than previously thought within both individual neurons and distinct brain regions. We only used antibodies and staining conditions which were verified on KO tissue, which may explain some of the localization differences we observed compared to previous studies.

5.2. Roles for the Localization and Local Translation of β-Actin

As discussed in Section 3.2, the localization and local translation of β-actin is one of the, if not the most, studied aspect of actin isoform biology. This is also true in neurons, where there is significant evidence for critical functions of the local translation of specifically β-actin in neuronal development, function, disease, and regeneration. It is worth noting, however, that the primary aim of these studies was to characterize and elucidate mechanisms mediating mRNA localization and local translation in general, while β-actin was most often used simply as a reporter for this process. In this section, we will focus our discussion on the evidence most directly relevant to specific actin isoform functions in neurons. The reader is also referred to excellent reviews elsewhere (Jung et al., 2012; Swanger and Bassell, 2011) for more in-depth discussions on the broader mechanisms of mRNA localization and local translation in developing neurons.

5.2.1. Local β-Actin Translation in Growth Cone Guidance

Following differentiation and migration, neurons in the developing CNS send out axons in search of specific synaptic targets. The task of ensuring the proper wiring of the nervous system falls to the axonal growth cone, which must interpret multiple soluble and cell-bound guidance cues to traverse sometimes vast distances in the developing CNS (Lowery and Van Vactor, 2009; Tessier-Lavigne and Goodman, 1996). The actin cytoskeleton is enriched in the peripheral domain of the growth cone, where localized asymmetric polymerization can initiate guided growth cone protrusion and turning. Given that the ultimate function of guidance cues is to provide

steering directions for elongating axons, it is not surprising that the final target of most if not all guidance signaling pathways is the modulation of the cytoskeleton, with actin being one of the primary targets (Dent et al., 2011). Guidance cue mediated regulation of actin-binding protein activity has been the predominant model used to explain localized actin polymerization within growth cones (Pak et al., 2008), but the high profile finding that β-actin can also be locally synthesized within growth cones likely adds another layer of complexity to that model.

Although we have found that β- and γ-actin proteins colocalize in growth cones (Cheever et al., 2012), only β-actin transcripts have been detected in growth cones to date (Bassell et al., 1998). Local translation of β-actin may thus subtly alter the composition of growth cone actin with important functional consequences. Interestingly, treatment with antisense oligonucleotides targeted to the β-actin mRNA zipcode sequence abrogated the guidance cue-induced enrichment of β-actin within growth cones, suggesting that a zipcode-ZBP1 mechanism may be involved (Zhang et al., 2001). Expression of a full length β-actin construct fused to GFP showed a similar neurotrophin-dependent increase in GFP localization within growth cones, while constructs lacking the zipcode sequence showed no enrichment of GFP localization. Since ZBP1 is known to be expressed in developing neurons (Zhang et al., 2001), the ZBP1-mediated localization and local translation of β-actin mRNA within growth cones may thus be an important mechanism in mediating actin reorganization and dynamics during growth cone guidance.

In 2006, two back-to-back studies published by independent groups using cultured *Xenopus* neuron systems tested the hypothesis that the local translation of β-actin is critical for growth cone turning and guidance. In the first study, β-actin mRNA and the *Xenopus* homolog of ZBP3/IMP3, Vg1 RBP, were found to colocalize within retinal neuron growth cones (Leung et al., 2006). Interestingly, the relative levels of Vg1 RBP and β-actin within growth cones could be enhanced upon exposure to the attractive guidance cue netrin-1. This localization appeared to result in increased local translation of β-actin mRNA, as netrin-1 treatment induced a significant increase in the amount of β-actin protein found within the growth cone by immunofluorescence. The increase in β-actin protein levels was blocked by protein synthesis inhibitors, and more interestingly, by antisense morpholinos directed at the start codon of β-actin to specifically block translation. Further evidence for the local translation of β-actin was presented when axons expressing a photoconvertible Kaede fluorescent protein fused to the 3′ UTR of β-actin were severed from their cell bodies. Following

permanent photoconversion of the preexisting reporter protein, neurons whose axon had been severed were exposed to netrin-1 and showed a significant increase in newly synthesized Kaede-β-actin reporter in growth cones, which was prevented by protein synthesis inhibitors or the expression of the reporter lacking the β-actin 3′ UTR zipcode sequence. Finally, the local translation of β-actin was examined in response to local gradients of netrin-1, which more closely mimic the guidance cue gradients neuronal growth cones encounter and interpret in vivo. β-actin protein was found to accumulate on the side of the growth cone proximal to the netrin-1 gradient, which could be blocked by treatment with protein synthesis inhibitors and more importantly the antisense morpholino predicted to block β-actin synthesis. It is interesting to note, however, that only a modest 23% decrease in β-actin protein was detected in growth cones following treatment with the morpholino. Nevertheless, that same morpholino completely blocked attractive turning of growth cones toward a gradient of netrin-1, while it had no effect on repulsive turning, demonstrating a specific, functional role for the local translation of β-actin in growth cones.

In the second study, *Xenopus* spinal neurons were used to demonstrate again that β-actin mRNA and ZBP3/VgRBP 1 colocalize in growth cones, which could also be enhanced upon treatment with the guidance cue brain-derived neurotrophic factor (BDNF) (Yao et al., 2006). This colocalization was significantly decreased by treatment with antisense morpholinos targeted against the zipcode sequence of β-actin however. Thus, this group chose to disrupt the interaction of β-actin mRNA with ZBP3/VgRBP 1 rather than block β-actin synthesis with morpholinos used by the first group. The two distinct approaches demonstrated similar effects however, as the second group also demonstrated that β-actin became preferentially enriched on the side of the growth cone proximal to a gradient of an attractive guidance factor. This finding on its own would not be entirely unexpected, but what makes it more interesting and substantial is that the asymmetrical distribution of β-actin was normalized to the distribution of γ-actin, which did not exhibit as significant an enrichment on the proximal side of the growth cone as β-actin. While it is well known that local actin accumulation initiates the membrane protrusion underlying growth cone turning, this was the first demonstration that β-actin *specifically* is locally enriched within the growth cone which does not appear to be the case of γ-actin, or at least not to the same extent. A similar significant asymmetric distribution of β-actin compared to γ-actin was also found to occur in growth cones encountering a repulsive cue, except this time with β-actin

accumulating on the side of the growth cone opposite to the gradient. And finally, treatment with antisense oligonucleotides to the β-actin zipcode sequence blocked attractive but also repulsive growth cone turning, which was in contrast to what was observed in the first report.

Collectively, these two prominent studies demonstrated that β-actin mRNA and protein accumulate asymmetrically on the side of the growth cone adjacent to an attractive guidance cue gradient, and that blocking this by either perturbing the interaction of β-actin mRNA with ZBP3 or blocking β-actin synthesis significantly decreased the ability of the growth cone to turn toward attractive guidance cues (and in one of the studies away from repulsive ones) (Leung et al., 2006; Yao et al., 2006). The strong data from these reports thus suggest that the localization and local translation of β-actin is a critical mechanism for mediating the localized accumulation of actin required for growth cone turning.

It remains controversial how well the local translation of β-actin is conserved in higher vertebrates, with data from some studies supporting its conservation while others have refuted it. Recent work characterizing cortical neurons cultured from ZBP1 KO mice demonstrated that ZBP1 is indeed required for attractive growth cone turning to netrin-1 and BDNF. However, β-actin protein accumulation in growth cones stimulated with BDNF was only very subtly decreased in the absence of ZBP1 (Welshhans and Bassell, 2011). This might suggest that other ZBP family members such as ZBP2 and 3 are able to compensate for the loss of ZBP1. Alternatively, the mislocalization of other RNAs localized by ZBP1 and critical for growth cone turning may be more severely affected than β-actin, the only RNA examined. As a more direct test of a role for the local translation of β-actin within mammalian axons, Vogelaar et al. cultured perinatal rat dorsal root ganglion (DRG) in compartmented chambers allowing for the specific manipulation of axons without affecting the neuronal cell bodies in a separate chamber. Treatment of axons with siRNAs against β-actin significantly reduced β-actin mRNA levels within axons, but not within the isolated neuronal cell bodies. Following axonal transection, control axons underwent a brief period of retraction followed by the reformation of a growth cone, while axons treated with the siRNA targeted against β-actin failed to reform a growth cone (Vogelaar et al., 2009). We recently demonstrated that β-actin-deficient motor axons functionally regenerate after peripheral nerve injury, however, suggesting that at least some axons are capable of regenerating in vivo in the complete absence of β-actin (Cheever et al., 2011).

Even more data contesting the relevance of local β-actin translation in growth cone guidance surfaced when it was demonstrated that local protein synthesis in total within axons was not required for growth cone turning responses to a variety of attractive and repulsive cues including ephrin-A2, slit-3, sema3A, NGF, and neurotrophin-3 (NT-3) (Roche et al., 2009). In contrast to the previous studies restricted to the *Xenopus* system, multiple neuronal subtypes and species were tested including chick retinal, sympathetic, DRG and mouse DRG neurons. This group showed that treatment with attractive guidance cues did induce a significant increase in the amount of F-actin within the growth cone by phalloidin staining, but that this was not dependent on protein synthesis and not due to an increase in β-actin protein levels based on immunofluorescence. Instead, β-actin was redistributed to the peripheral domain suggesting that the β-actin protein already present is sufficient for guided growth cone motility. RhoA, another protein previously reported to be locally translated within growth cones in response to guidance cues, was also not found to be locally translated in this study as well, further confirming that local translation within axons is not required for turning responses at least in the neuronal types examined.

One intriguing explanation offered by this group as to why their results differed from what has been reported by others is that the energy and metabolic demands of the various types of neurons examined across different species may differ. Based upon the estimated number of ribosomes within a growth cone, the translation rate of an actin monomer, and the magnitude of the increased β-actin synthesis observed in response to netrin-1, *Xenopus* growth cones would likely contain 100 times less β-actin than chick DRG growth cones for example (Roche et al., 2009). Dramatically different levels of β- and γ-actin in *Xenopus* spinal neurons as compared to chick DRG neurons was later directly demonstrated with multiple antibodies including some validated on β- and γ-actin KO tissue (Marsick et al., 2010). Additionally, *Xenopus* neurons were found to have a decreased F-:G-actin ratio compared to chick DRG neurons, which could have significant effects on actin dynamics and possibly even gene expression (Marsick et al., 2010). Thus, these studies raise the possibility that the local translation of β-actin may be essential for proper growth cone turning in *Xenopus* neurons, which express actin at much lower levels than in higher vertebrate growth cones where the local synthesis of β-actin does not appear to be required.

The role for local translation in axons and growth cones remains contentious, however, and more clarity would likely be provided with in vivo studies to complement the predominantly in vitro work performed so far. We recently generated a CNS-specific β-actin KO mouse (CNS-*Actb*KO), where β-actin protein levels were rapidly depleted from the embryonic mouse brain (Cheever et al., 2012). Interestingly, we found normal positioning and morphology of the anterior commissure and rostral corpus callosum, suggesting that axon elongation and guidance can occur normally in the absence of local translation of β-actin. Yet, we cannot rule out the possibility that preexisting β-actin mRNA and protein may be sufficient to facilitate the predominantly normal axonal development observed. However, we feel several pieces of evidence strengthen the suitability of the CNS-*Actb*KO model. First, most of the large axonal tracts we examined develop relatively late embryonically, starting around E14.5–15.5 (Rash and Richards, 2001). Importantly, in whole brain western blots from CNS-*Actb*KO embryos, we observed a 50% decrease in β-actin protein levels just 3 days after Cre recombinase expression at E13.5 (Cheever et al., 2012), suggesting that preexisting β-actin mRNA and protein were exhausted rapidly to allow for protein turnover. Furthermore, full length β-actin mRNA transcripts would have ceased to be made even prior to this with Cre expression at E10.5 based on the gene targeting scheme employed (Perrin et al., 2010).

While most of the axonal tracts examined appeared to have developed normally, there was one striking exception. The caudal portion of the corpus callosum failed to cross the midline in CNS-*Actb*KO brains (Cheever et al., 2012). What is intriguing about this phenotype is that the axons of the rostral corpus callosum, which were able to navigate successfully, have an arguably more complex path to follow at roughly the same developmental timepoint. Pioneer axons of the rostral corpus callosum must interpret guidance cues and gradients established by a number of different glial and support cell populations to successfully reach the midline, and then extend beyond it to reach their synaptic targets in the contralateral side of the brain (Lindwall et al., 2007; Richards et al., 2004). In the caudal portion of the corpus callosum, however, it is believed that axons simply follow a preexisting tract of axons that forms 1 day earlier (Livy and Wahlsten, 1997; Richards et al., 2004). This preexisting tract lies immediately ventral to the corpus callosum and is known as the *dorsal hippocampal commissure*, composed of hippocampal neuron axons crossing the brain to synapse with the contralateral hippocampus. A netrin-1 gradient localized to the midline of the

brain is believed to be one of the primary guidance cues that guide axons of the hippocampal commissure to the other side of the brain (Barallobre et al., 2000; Serafini et al., 1996; Steup et al., 2000).

Because the dorsal hippocampal commissure is also absent in CNS-*Actb*KO mice, we hypothesized that the axons of the corpus callosum lacked their normal growth substrate and sought to determine if the hippocampal neurons exhibited an impaired response to netrin-1. To accomplish this, we took advantage of the previously described increase in hippocampal neuron axonal branching following exposure to netrin-1 as a readout for axonal responsiveness (Popko et al., 2009). Axonal branching is relatively simple to measure and shares many common features with growth cone turning including localized actin polymerization and Ca^{2+} signals (Dent et al., 2003, 2004), although there are differences as well. Using this axonal branching paradigm, we found that neurons devoid of β-actin protein by immunofluorescence (Cheever et al., 2012) exhibited a similar increase in axonal branching to netrin-1 as compared to controls (Fig. 4.3), suggesting that downstream signaling from the netrin-1 receptor deleted in colorectal cancer does not require local β-actin translation. Why β-actin–deficient axons of the hippocampal commissure fail to cross the midline is still not entirely clear, although we favor the hypothesis that disruptions in the local hippocampal architecture (Section 5.1.2) likely play a prominent role since only axons extending through this region appear perturbed. If β-actin was

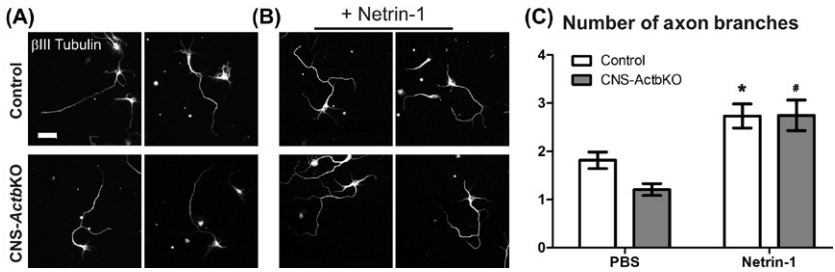


Figure 4.3 ***Netrin-1 induced axonal branching in control and β-actin KO hippocampal neurons.*** (A–B) Representative images of βIII-tubulin-stained primary hippocampal neurons cultured from control and CNS-*Actb*KO embryos. Neurons were treated with a bath application of either PBS or 250 ng/ml netrin-1 for 3 days followed by fixation, staining and analysis. (C) β-actin-deficient neurons showed a comparable increase in axonal branching in response to netrin-1 as control neurons. * denotes significantly different than control neurons treated with PBS. # indicates significantly different than β-actin KO neurons treated with PBS. $n > 100$ neurons for each genotype from at least two independent experiments. Scale bar 20 μm. Representative control images for comparison from Cheever et al. (2012).

required for growth cone guidance, we would have expected to see a similar defect in the rostral corpus callosum, which crosses the midline at approximately the same time (Richards et al., 2004).

In summary, strong data from cultured *Xenopus* neuron systems indicating that the asymmetric local translation of β-actin is required for growth cone guidance have not been definitively confirmed in higher vertebrate cell culture or in vivo models. This is significant because β-actin is the most commonly cited and utilized reporter for local translation in growth cones, as well as one of the most commonly cited paradigms for the functional relevance of local translation. ZBP1 appears to be required for growth cone guidance in mammalian neurons in vitro, however, and so it remains possible that the localization and local translation of any of the other several hundred transcripts shown to interact with ZBP1 could be contributing to growth cone guidance while the association with β-actin could simply be due to a nonfunctional bystander effect. Caution should thus be employed when extrapolating function to the localization of β-actin mRNA and protein at least within growth cones of higher vertebrates at this time.

5.2.2. *Local Translation of β-Actin in Dendrites and Dendritic Spine Morphogenesis*

Local translation within developing and mature dendrites is generally more widely accepted than local protein synthesis in growth cones due to the prohibitively large distances proteins would need to be transported in mature neurons versus the relatively shorter distances in developing neurons (Bramham, 2008; Job and Eberwine, 2001; Sutton and Schuman, 2006). Furthermore, a large number of these studies have implicated local protein synthesis as a critical factor modulating synaptic plasticity. Although ZBP1 levels decrease substantially with age in mice (Nielsen et al., 1999; Runge et al., 2000), a small number of studies have now identified endogenous ZBP1 expression in the dendrites of mature cultured neurons (Eom et al., 2003; Perycz et al., 2011; Tiruchinapalli et al., 2003), raising the possibility that the regulated local translation of β-actin could play a role in dendrite development or synaptic plasticity. These few studies have likely just scratched the surface of potential actin isoform functions in dendrites, however, and given the significant memory and behavioral defects identified in CNS-*Actb*KO mice, we feel this area is among the most promising and exciting that remains to be explored in actin isoform neurobiology.

One recent study examined the role of ZBP1 and β-actin localization in regulating the dendritic morphology of cultured hippocampal neurons.

Using multiple different shRNA vectors to knock down ZBP1 expression, it was found that ZBP1 appears to be required for the development but not maintenance of dendritic branches (Perycz et al., 2011). This effect was dependent on the RNA-binding and Src-mediated derepression functions of ZBP1, strongly suggesting that localization of ZBP1 target mRNAs is critical for proper dendritic arborization. These authors then proceeded to examine the distribution of β-actin mRNA and protein within control and ZBP1 knockdown neurons. They found that β-actin levels decreased significantly only in distal, but not proximal dendrites, providing support for the hypothesis that local translation of proteins and specifically actin is most crucial at large distances from the neuronal cell body. Overexpression of β-actin partially rescued the decreased dendritic branching phenotype in ZBP1 knockdown neurons, although by what mechanism and if the zip-code sequence was included in the vector were not clear. These results are also consistent with what has been observed in β-actin-deficient primary hippocampal neurons. Although not statistically significant, β-actin KO neurons also showed a trend of decreased dendritic branching at a much earlier timepoint in culture (Cheever et al., 2012). Interestingly, overexpression of microtubule-associated protein 2 (MAP2) also rescued some of the phenotypes associated with ZBP1 knockdown, suggesting that impaired localization of β-actin may not be exclusively responsible for the simplified dendritic branching (Perycz et al., 2011). Further work will be required in order to determine if β-actin is definitively involved in dendritic morphology, or perhaps merely a bystander.

Beginning at roughly the same time as dendritic arbor maturation, filopodia strung along the length of dendrites begin to form, elongate, and retract in an apparent stochastic nature (Holtmaat and Svoboda, 2009; Hotulainen and Hoogenraad, 2010; Yoshihara et al., 2009). However, when these filopodia encounter an axon, they can become stabilized most likely due to electrical or trophic signals, which elicit a stabilization of the underlying actin cytoskeleton (Shimada et al., 1998; Tiruchinapalli et al., 2003). Further increases in actin polymerization can result in the tip of the filopodia expanding, creating a mushroom-like morphology characteristic of a mature dendritic spine, the site of the majority of excitatory synapses in the mammalian brain (Honkura et al., 2008; Hotulainen and Hoogenraad, 2010; Okamoto et al., 2004). With an ever increasing number of studies identifying perturbed numbers or morphology of dendritic spines in various neurodegenerative and neurodevelopmental disorders (Fiala et al., 2002; Penzes et al., 2011), the mechanisms by which dendritic spines are formed is

currently an area of intense investigation. Given the critical roles of actin in dendritic spine formation and function, there is significant potential for isoform specific roles during these processes (Fukazawa et al., 2003; Honkura et al., 2008; Hotulainen and Hoogenraad, 2010).

Dendritic spines and growth cones intriguingly share a large number of similarities, with both largely dependent on the actin cytoskeleton and its dynamics for the morphological changes required for proper functioning. Given the critical role for ZBP1 in growth cone function, and that it also appears to be expressed in mature cultured neurons, it is not entirely surprising that two studies have characterized apparent roles for ZBP1 in the formation and regulation of dendritic filopodia, the precursors of spines. β-actin mRNA and ZBP1 were found to colocalize in small granules along the dendrites of cultured hippocampal neurons, and interestingly, this colocalization could be enhanced following depolarization with KCl (Tiruchinapalli et al., 2003). Similar to the study described above, knockdown of ZBP1 resulted in a significant decrease in the dendritic localization of β-actin mRNA and also reduced the density of dendritic filopodia under both basal and BDNF stimulated conditions (Eom et al., 2003). Overexpression of a full length β-actin construct with the zipcode sequence also increased the density of dendritic filopodia as compared to expression of a β-actin construct lacking the zipcode sequence (Eom et al., 2003).

Collectively, these studies suggest that activity-regulated recruitment of β-actin mRNA by ZBP1 may be an important mechanism initiating dendritic spine morphogenesis, potentially by promoting the local translation and accumulation of β-actin. It is interesting to note, however, that expression of epitope-tagged β- and γ-actin constructs were both strongly localized to dendritic spines in the absence of the zipcode sequence (Kaech et al., 1997). Thus, as has been postulated by Welshhans et al. in growth cones (Welshhans and Bassell, 2011), ZBP1-mediated localization of β-actin to dendritic filopodia and spines may only be required or functionally relevant under certain conditions such as in response to synaptogenic signals or synaptic stimulation. This, of course, is also dependent on ZBP1 being expressed by mature neurons in vivo, which remains to be demonstrated.

Distinct functions for actin isoforms in mature dendritic spines, especially as it relates to the encoding of memory and synaptic plasticity, represent an incredibly exciting and completely unexplored aspect of actin isoform function in neurons. Learning and memory are postulated to be encoded in neural networks by either changes in the strength of synapses or the addition and/or elimination of existing ones (Lamprecht and LeDoux, 2004).

Long-term potentiation (LTP) is one means of increasing the strength of existing synapses and is currently believed to be a molecular mechanism underlying the encoding of memory. In experiments with cultured neurons and in vivo, stimulation of neurons leading to the induction of LTP is strictly dependent upon an increase in actin polymerization within spines, occurring within minutes of stimulation and generally leading to an increase in spine volume and insertion of neurotransmitter receptors (Fukazawa et al., 2003; Honkura et al., 2008; Matsuzaki et al., 2004; Okamoto et al., 2004). High magnitude but transient intracellular Ca^{2+} signals are also critical for the induction of LTP in dendritic spines (Oertner and Matus, 2005). While the functions of this Ca^{2+} signal most certainly affect actin dynamics indirectly through the modulation of actin-binding proteins, it is also tempting to speculate that this could more directly affect actin polymerization (specifically β-actin) by elevating the levels of Ca^{2+} bound β- and γ-actin. Intriguingly, multiple populations of actin with distinct localizations and turnover rates have now been observed in dendritic spines at rest and in those stimulated by glutamate to mimic synaptic transmission (Honkura et al., 2008). It is thus tempting to speculate that microgradients of Ca^{2+} adjacent to channels within the plasma membrane may lead to the manifestation of the distinct polymerization kinetics of Ca^{2+} bound β- and γ-actin, adding another layer to the complexity of postsynaptic actin regulation. Future studies examining the subspine distribution of actin isoforms as well as exploring how β- and γ-actin null neurons may respond differentially to Ca^{2+} signals during LTP will likely shed light on this unexplored topic.

6. INSIGHTS INTO ACTIN ISOFORM FUNCTIONS FROM IN VIVO STUDIES AND HUMAN DISEASES

Thus far we have reviewed the roles and regulation of actin isoforms as it pertains to neuronal development and function. It is important to note, however, that much of this data were acquired in cell culture systems and often revealed relatively subtle roles for actin isoforms, making it difficult to infer what significance the observed effects may have for neuronal development and function within the nervous system of an intact organism. Recently, in vivo mouse models have begun to be generated and used to assess the physiological significance of the many in vitro roles for β- and γ-actin. Based on the functions we discussed in the previous section, if β-actin plays similar roles within mammalian neurons in vivo, one might predict that a mouse lacking β-actin expression would have profound and

severe phenotypes relating to neuronal migration, axon guidance, and synaptic function. In this section, we will discuss what has been learned from a number of mouse models as well as human studies relating to the roles of β- and γ-actin in neuronal development and function.

6.1. Novel Roles for β-Actin Revealed by a CNS-Specific β-Actin Knockout Mouse

6.1.1. Contributions of β-Actin to Actin Isoform Expression within the Brain

Ablation of an actin isoform has now been demonstrated in several different tissues and cell types, and intriguingly almost always results in the maintenance of total actin levels by upregulation of alternative actin isoforms (Belyantseva et al., 2009; Bunnell and Ervasti, 2010; Bunnell et al., 2011; Cheever et al., 2012). Although whole-body *Actb*KO mice exhibit early embryonic lethality (Bunnell et al., 2011; Shawlot et al., 1998; Shmerling et al., 2005), we found that β-actin was rapidly ablated in the brains of CNS-*Actb*KO embryos while total actin levels were preserved during development (Cheever et al., 2012). The maintenance of total actin levels was due to a modest upregulation of γ-actin and a significantly larger increase in the more distantly related α-smooth actin. Why α-smooth actin was upregulated to such a significant extent is not clear. α-smooth actin mRNA contains no identifiable zipcode sequence, and the protein is classically used as a marker for myofibroblasts, which migrate into and facilitate the closure of wound sites (Darby et al., 1990). The amount of α-smooth actin in a cell culture model of myofibroblasts has also been shown to be directly proportional to the amount of traction force a cell is able to generate (Chen et al., 2007; Hinz et al., 2002). It is thus tempting to speculate that neurons may specifically upregulate α-smooth actin in the absence of β-actin due to its cell traction producing properties, which would certainly be useful for migrating neurons in vivo. Nevertheless, it also possible that the ectopic expression of α-smooth actin is itself pathogenic and responsible for some or all of the phenotypes observed in CNS-*Actb*KO mice. Future studies with β-α-smooth actin double KO mice will be required to determine if the upregulation of α-smooth actin is compensatory, detrimental, or has no effect. Additionally, β-γ-actin double KO mice could provide insight into whether the upregulation of γ-actin is also functionally relevant.

The preservation of total actin levels during development may explain why CNS-*Actb*KO embryos were morphologically indistinguishable from controls at birth, although nearly two-thirds were still lost perinatally

(Cheever et al., 2012). The reason or reasons for the perinatal lethality are not clear, although even subtle behavioral or other differences between β-actin KO and control pups could result in the KO pups being ignored or even cannibalized. Those CNS-*Actb*KO pups that did survive were about two-thirds the size of control littermates despite the fact that they had a significantly higher caloric intake. Interestingly, this is very similar to what was observed in the inducible *Ate1*KO mice (Brower and Varshavsky, 2009), raising the possibility that the loss of arginylated β-actin is a factor in this phenotype. Both mouse models exhibited profound hyperactivity and despite an increased caloric intake, still exhibited stunted growth. Impaired hypothalamic function and regulation of hunger could also be a factor in both of these mouse models, although hypothalamic function was not directly investigated in CNS-*Actb*KO mice. Yet, in contrast to the *Ate1* KO mouse model, CNS-*Actb*KO brains were proportionally smaller than control littermates, thus confirming that *Ate1* has significant functions beyond just the arginylation of β-actin.

6.1.2. Restricted Roles for β-Actin in Mediating Brain Structure

As stated in the introduction to this section, with the widely held hypothesis that β-actin specifically regulates directional cell migration, it could be predicted that histological examination of the brains from CNS-*Actb*KO mice would reveal profound disorganization given the large amount of cell migration required to properly assemble the brain. Thus, we were quite surprised to find that the vast majority of the brain in CNS-*Actb*KO mice was histologically indistinguishable from controls (Cheever et al., 2012). Proper cell layering in CNS-*Actb*KO brains could be seen in the normal organization of the molecular and granule cell layer in the cerebellum, in the pyramidal neuron layers in the hippocampus, as well as in the six cortical layers of the cortex. Collectively, these data suggest that most if not all neuronal migration does not require β-actin. However, because the migration of some neurons is completed relatively early in development (Ayala et al., 2007), it cannot be ruled out that the low levels of β-actin still present soon after Cre-mediated recombination were sufficient to drive normal neuronal migration. Given that the Nestin-Cre line used is one of the earliest lines available for neuronal specific expression, technological advances will likely be required to definitively rule out a role for β-actin in early neuronal migration.

In our analyses of the cerebellum and hippocampus, however, we did observe striking yet highly localized morphological abnormalities. In the

cerebellum, for example, we found variable abnormal organization or branching of the cerebellar folia (Fig. 4.4, asterisks). Within the hippocampus, significant displacement of the dentate gyrus and hippocampal fissure was observed resulting in decreased CA1 cell density localized to the dentate gyrus/hippocampal fissure invagination (Fig. 4.4 red arrows and asterisks). Intriguingly, the cerebellum and hippocampus undergo somewhat similar developmental programs during morphogenesis, which may shed light on the mechanisms underlying the specific abnormalities observed. The developing cerebellum, for example, starts off as a relatively smooth, elongated sphere in the mouse hindbrain. Later, a select population of cells is thought to attach to the extracellular matrix (ECM) or another cell type, thereby forming hingepoints through which the developing cerebellar folia grow out from (Sudarov and Joyner, 2007). The abnormal positioning and organization of the cerebellar folia in CNS-*Actb*KO mice suggests that β-actin may perform a specific function in the regulation of hingepoint specification and positioning, potentially by mediating cell–ECM or cell–cell adhesions.

The morphological abnormalities observed in the hippocampus may also share a common mechanism with those described in the cerebellum. The developing hippocampus begins essentially as a thin sheet of cells where select anchor points are established causing the hippocampus to fold in on itself as it enlarges, creating the hippocampal fissure and its recognizable interlocking "C" morphology (Eckenhoff and Rakic, 1984; Smart, 1982). Histological data from CNS-*Actb*KO mice suggest that the folding of the hippocampus occurs abnormally in the absence of β-actin, leading to the displacement of the hippocampal fissure, dentate gyrus, and adjacent CA1 region (Fig. 4.4) likely via impaired regulation of cell–ECM or cell-adhesions.

There may also be an even more direct connection between a specific role for β-actin and the cellular constriction that promotes tissue bending during brain morphogenesis. Tissue bending similar to what occurs during the development of the cerebellum and hippocampus is perhaps best modeled by neural tube closure, where cells at the hingepoints of the neural tube undergo an actin and myosin-mediated apical constriction causing adjacent cells to be pulled inward and bending the tissue (Copp et al., 2003; Martin, 2010). Interestingly, β-actin has been found to be specifically enriched at the cleavage furrow of dividing cells in culture, suggesting that β-actin within the actin–myosin contractile rings may be particularly important for cellular constriction during cell division and tissue bending

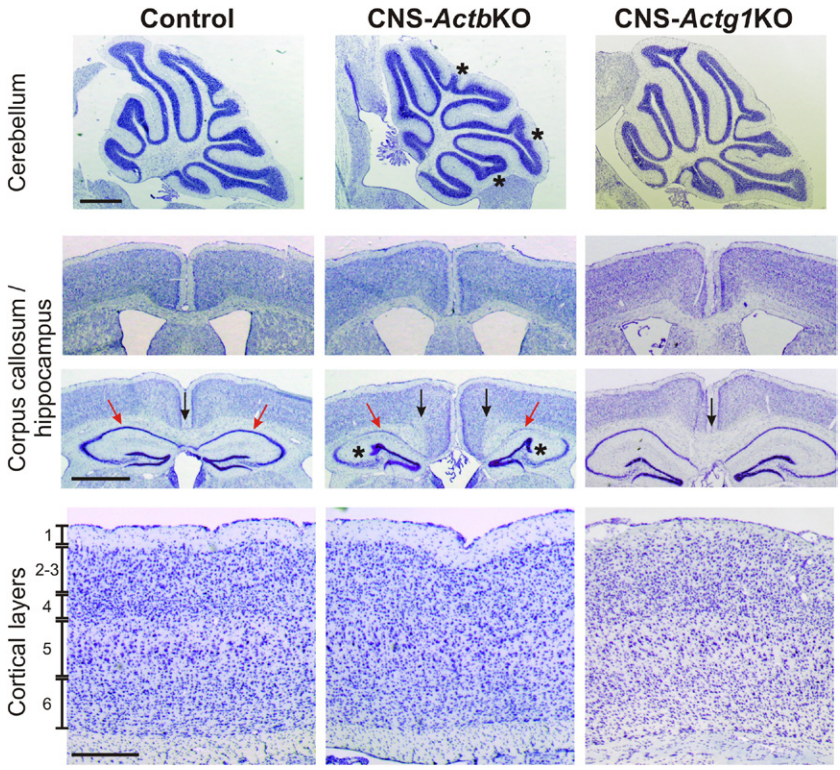


Figure 4.4 ***Histological phenotypes following ablation of β- but not γ-actin in the brain.*** Midsagittal sections through the cerebellum of CNS-*Actb*KO mice revealed abnormalities in the positioning and morphology of the cerebellar folia (asterisks) that was not observed in CNS-*Actg1*KO animals. Scale bar 0.5 mm. Coronal sections through the rostral portion of the brain (second panel from top) showed the normal crossing of the rostral corpus callosum in all genotypes. However, a localized defect in corpus callosum crossing was observed in CNS-*Actb*KO mice in more caudal sections overlying the hippocampus (black arrows in third from top panel). An abnormal invagination of the dentate gyrus (asterisks) and decreased CA1 staining (red arrows) was also only apparent in CNS-*Actb*KO animals. Scale bar 1 mm. Sagittal sections through the cortex (bottom panel) revealed no significant defects in cortical layering in either CNS-*Actb* or *Actg1*KO brain sections. Scale bar 0.3 mm. Control and CNS-*Actb*KO images for comparison from Cheever et al. (2012). For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

(Dugina et al., 2009). Cells depleted of β-actin by RNAi-mediated knockdown or genetic ablation both exhibit impaired growth and significantly elevated numbers of multinucleate cells while γ-actin-deficient cells do not (Bunnell et al., 2011; Dugina et al., 2009), further supporting the idea that β-actin is specifically involved in cellular constriction events. Although

Nestin-Cre expression initiates just as the neural tube is closing, the fact that whole body *Actg1* KOs and CNS-*Actg1*KO mice do not exhibit any neural tube or similar brain morphological defects (Bunnell and Ervasti, 2010 and Fig. 4.4) supports the model of β-actin-specific functions in brain morphogenesis.

6.1.3. β-Actin Contributes to the Proper Regulation of Select Behaviors

In order to determine whether β-actin specifically contributes to normal brain function, we analyzed the behaviors of CNS-*Actb*KO mice using a number of different experimental paradigms. Given the significant disruption of the hippocampus, we started by subjecting control and CNS-*Actb*KO mice to the Morris water maze, which is highly dependent on hippocampal function (Morris et al., 1982; Tsien et al., 1996; Vorhees and Williams, 2006). We found that mice lacking β-actin within the brain had significant impairment in water maze performance, with data suggesting that CNS-*Actb*KO mice actually actively avoided escape from the maze despite a nearly normal training phase (Cheever et al., 2012). This observation raises the possibility that the mice failed to learn the goal of the test (to find the hidden platform), or that perhaps these mice had heightened anxiety potentially confounding their spatial memory performance. We also employed an open field activity assay, which can shed light on hippocampal and anxiety-mediated behavioral abnormalities (Mignogna and Viggiano, 2010; Viggiano, 2008). CNS-*Actb*KO mice were found to be profoundly hyperactive, and to such an extent that it interfered with interpretation of anxiety readouts from this assay. While this hyperactive phenotype could be due to large number of mechanisms, we did rule out that the absence of β-actin perturbs the regulation of circadian rhythm-mediated activity cycles which were normal in CNS-*Actb*KO mice (Fig. 4.5).

At present, it is unclear whether the morphological abnormalities noted in the brains of CNS-*Actb*KO mice are directly responsible for the behavioral phenotypes just described. Although the invagination of the dentate gyrus and hippocampal fissure cuts across the normal path of the Schaffer collateral axons in the hippocampus for example, it is possible that the axonal tracts may be mislocalized but still intact, thus preserving the integrity of the hippocampal circuit. Given the critical role for actin and potentially the local translation of β-actin at dendritic spines, it would be intriguing to utilize the recently described conditional β-actin KO mouse to ablate β-actin after hippocampal morphogenesis is complete (Zhang et al., 2012). Upon confirming that the hippocampus is

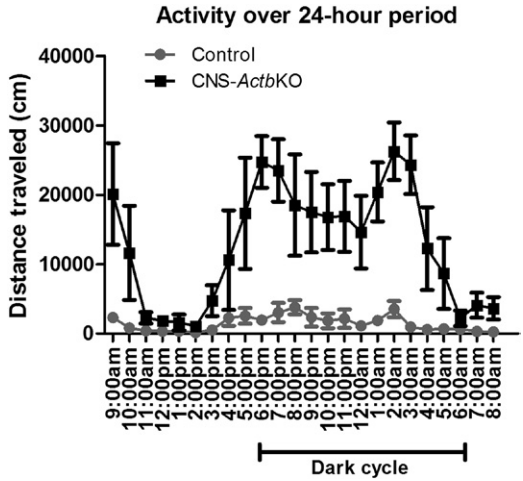


Figure 4.5 ***Activity levels of CNS-ActbKO mice over a 24-h period.*** CNS-*Actb*KO mice were significantly more active than control mice over a 24-h period but did show activity patterns consistent with a normal circadian rhythm. Data represent mean ± standard error of the mean. $n = 4$ mice per genotype.

indeed histologically normal, the relatively simple and high-throughput open field activity assay could be performed to ascertain the relative extent that the large-scale morphological abnormalities contributed to the functional effects observed in CNS-*Actb*KO mice. In addition, the use of the CNS-*Actg1*KO mouse model, which does not exhibit hippocampal or any other morphological brain abnormalities, could provide an additional control. The normal development of the hippocampus but persistence of behavioral phenotypes would suggest that localized defects at synapses and perhaps dendritic spines may be the primary mechanism behind the observed behavioral abnormalities in the absence of β-actin.

Perhaps the most surprising behavioral defect observed in the CNS-*Actb*KO mice was a profound maternal behavior deficit. No pup born to a CNS-*Actb*KO mother survived for longer than 1 day regardless of genotype, which also correlated with an apparent pup retrieval defect (Cheever et al., 2012). Neonatal mice are unable to completely regulate internal body temperature, and are thus dependent on retrieval into a nest to maintain body temperature. The CNS-*Actb*KO pup retrieval deficit was independent of the pups, as the same litter was retrieved and cared for normally when fostered to a control mother. The specific mechanism or mechanisms underlying this phenotype are at present unclear, although there are a number of intriguing possibilities.

Maternal behavior abnormalities, including pup retrieval deficits similar to what we observed in CNS-*Actb*KO mice, have been reported in a number of different transgenic mouse models including the transcription factor FosB (Brown et al., 1996) and the tubulin interacting protein stathmin (Martel et al., 2008). Research into these and other models has begun to elucidate some of the primary neural circuits responsible for proper maternal behavior. One of the most well-studied brain regions implicated in maternal behavior includes the medial preoptic area (MPA) of the hypothalamus and the adjacent ventral bed nucleus of the stria terminalis (Gammie, 2005; Leckman and Herman, 2002; Numan, 2007). For simplicity, we will refer to this region collectively as the MPA. Lesion studies designed to cut off neural circuits in and out of the MPA result in the complete loss or severe disruption of pup retrieval and nesting behaviors (Gammie, 2005; Numan, 2007). Interestingly, the same brain region has also been implicated in the regulation of social, ingestive (eating and drinking), and general exploratory or foraging behaviors (Leckman and Herman, 2002). CNS-*Actb*KO mice also presented with ingestive and exploratory behavioral abnormalities, further suggesting that hypothalamus function may be disrupted in the absence of β-actin. Whether the impairment of function is due to more wide-scale disruption of major axonal and dendritic tracts or more local disruption at individual synapses and dendritic spines remains to be determined. It is known, however, that the onset of maternal behavior occurs concomitantly with significant reprogramming and plasticity in the brain of pregnant females (Gammie, 2005; Numan, 2007). For example, at the onset of maternal behavior, studies have demonstrated that mothers find their pups more rewarding cocaine (Mattson et al., 2001). However, after approximately 3 weeks or when mouse pups are normally weaned, this "addiction" to the pups is lost, making maternal behavior studies relevant to other behavioral paradigms including addiction. It will be intriguing to determine if CNS-*Actb*KO mice show any resistance to the effects of addictive drugs, providing novel insight into both the mechanisms and potential therapeutic targets for overcoming addiction in humans.

Another intriguing mechanism that could contribute not only to the maternal behavior deficit in CNS-*Actb*KO mice but also to the abnormal water maze performance and hyperactivity relates to adult neurogenesis. In the last decade or so following the acceptance that new neurons are indeed generated throughout life albeit to a limited extent, an explosion of studies has begun to uncover highly significant roles for adult neurogenesis (Deng et al., 2010; Ming and Song, 2005; Moreno et al., 2009). In

mice, as in humans, adult neurogenesis is confined to the subventricular zone and the dentate gyrus of the hippocampus (Lledo et al., 2006). New neurons generated within the subventricular zone migrate along a defined path composing the rostral migratory stream to the olfactory bulb where they integrate into existing circuits (Alvarez-Buylla and Garcia-Verdugo, 2002). The functional role that these neurons play has been somewhat controversial, although some studies have demonstrated that these new neurons facilitate odor discrimination and olfactory memory (Enwere et al., 2004; Gheusi et al., 2000). Interestingly, adult neurogenesis in the subventricular zone increases significantly during late pregnancy in mice (Shingo et al., 2003), raising the possibility that these new neurons could play a role in pup recognition, a critical factor leading to the proper retrieval and onset of maternal behaviors. In the dentate gyrus of the hippocampus, the function of adult neurogenesis is similarly controversial, but has been shown to correlate positively with hippocampal-dependent learning paradigms (Clelland et al., 2009; Deng et al., 2010; Shors et al., 2001).

Given that the loss of β-actin has recently been demonstrated to severely impair cell growth and division, and that newly generated neurons in adults must migrate sometimes significant distances to integrate properly into synaptic circuits, it is enticing to speculate that adult neurogenesis may also be perturbed in CNS-*Actb*KO mice. Early experiments have not provided conclusive evidence yet, however. Despite a peculiar response to water, CNS-*Actb*KO mice exhibited normal olfactory discrimination, a behavior linked to adult neurogenesis (Fig. 4.6). However, the abnormal behavior exhibited by CNS-*Actb*KO mice in the Morris water maze could potentially be due to disrupted adult neurogenesis in the dentate gyrus. More definitive experiments remain to be conducted, including BrdU labeling in adult animals to directly assess the number and localization of newly generated neurons in CNS-*Actb*KO brains. Another particularly useful tool would be to cross the conditional β-actin allele to an inducible Nestin-Cre^{ER} transgenic line, which is expressed only after induction in neural progenitors in adult animals (Chen et al., 2009). Such an experiment would provide additional direct evidence for a role of β-actin in adult neurogenesis and associated behaviors.

6.2. Insight from ZBP1 Knockout and Heterozygous Mouse Studies

If ZBP1 is critical for the localization and function of β-actin in neurons in vivo, one might expect that a ZBP1 KO mouse would phenocopy or exhibit even a more severe phenotype than CNS-*Actb*KO mice due to the

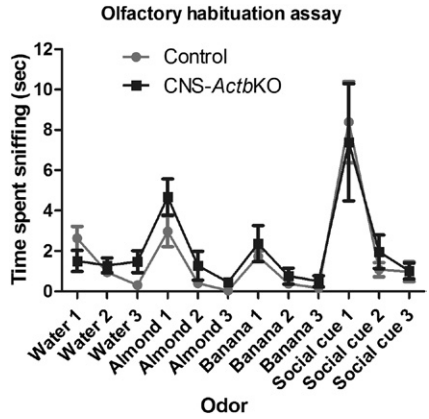


Figure 4.6 ***Analysis of olfactory discrimination in CNS-ActbKO mice.*** Control and CNS-*Actb*KO mice were exposed to novel and social odor cues in three consecutive trials to assess the rate of olfactory habituation. Following the third trial, mice were exposed to the next cue to measure dishabituation and recognition of a novel odor. CNS-*Actb*KO mice showed similar habituation and dishabituation to novel and social odor cues compared to controls. However, CNS-*Actb*KO mice did not exhibit dishabituation to the water control suggesting they were unable to smell this cue or had a heightened attraction to it. Data represent mean ± standard error of the mean. $n = 9$ mice per genotype.

mislocalization of other ZBP1 targets. A ZBP1/IMP1 KO mouse generated by gene trap insertion has indeed been characterized, and interestingly, exhibits significant perinatal lethality and stunted growth similar to CNS-*Actb*KO mice (Hansen et al., 2004). However, these phenotypes are relatively nonspecific and commonly found in many diverse transgenic and KO mouse lines. Aside from noting a proportionally reduced brain size in IMP1 KO mice, no brain pathology was reported although it is unclear to what extent brain histology was actually analyzed (Hansen et al., 2004). It was discussed, however, that the few surviving IMP1 KO mice exhibited restlessness and circular movements, perhaps indicating neurobehavioral abnormalities. The most prominent histological phenotypes noted included a reduction in primary fibroblast proliferation, reduced size of intestinal microvilli, and less prominent malformations of the small intestine, colon, kidney and liver. It was concluded that the abnormalities in the latter organ systems likely caused the significant perinatal lethality in the IMP1 KO mice.

Some insight into potential neuronal functions of ZBP1 in vivo was recently acquired when a report characterized DRG development and sciatic nerve regeneration in heterozygous ZBP1 mice (Donnelly et al., 2011). This group demonstrated that overexpression of the β-actin 3′ UTR fused to GFP can decrease the axonal localization of endogenous β-actin mRNA,

which also correlated with decreased axonal outgrowth in cultured DRG neurons. It was inferred that the β-actin 3′ UTR-GFP construct can saturate the cellular mRNA localization machinery (presumably ZBP1), and lead to deficits in mRNA localization and local translation. It was further shown that forced overexpression of ZBP1 could rescue this phenotype, suggesting that ZBP1 may actually be a limiting factor in RNA localization and axon outgrowth. Heterozygous ZBP1 mice express approximately 40% of the ZBP1 mRNA found in wild-type littermates, and interestingly exhibit similar axon outgrowth deficits as those observed in neurons expressing the β-actin 3′ UTR-GFP construct as well as impaired sciatic nerve regeneration in vivo. Overexpression of β-actin does not rescue the effects of limited ZBP1 availability, however, consistent with the finding that β-actin is not required for functional nerve regeneration in vivo (Cheever et al., 2011). Thus, as noted above, ZBP1 likely plays an important role in neuronal development and function but evidence suggesting that this function is primarily through the localization of β-actin mRNA and protein in vivo remains elusive. While the β-actin 3′ UTR and full length mRNA appear to be ideal readouts for ZBP1 function, care should be taken in inferring that this has direct physiological relevance in vivo.

6.3. Human Neurological Diseases Related to β- and γ-Actin

The specific contributions of actin isoforms to human neuronal function and disease have only recently begun to receive attention. However, in the last two decades, mutations in both genes encoding β- and γ-actin (*Actb* and *Actg1*, respectively) have been identified in a small group of patients often exhibiting neurological phenotypes. Broadening the search parameters of human diseases to include conditions where an actin isoform is believed to be specifically misregulated reveals the possible connection between β-actin and the motor neuron disease spinal muscular atrophy (SMA). Implications for actin isoform roles in these human conditions are described below.

6.3.1. Potential Role for β-Actin in Spinal Muscular Atrophy

SMA is the leading genetic cause of death in infants and the second most common autosomal recessive disease in humans with an incidence of 1 in 10,000. Mutations in the survival motor neuron 1 gene (*SMN1*) lead to decreased levels of its encoded protein product SMN, resulting in the loss of lower α-motor neurons, muscle atrophy, and in the most severe cases, death (Lefebvre et al., 1995; Lunn and Wang, 2008; Monani, 2005). SMN is ubiquitously expressed with functions best understood in the biogenesis

and assembly of snRNPs mediating mRNA splicing (Monani, 2005). Why decreased levels of SMN leads selectively to the loss of motor neurons is a question that has plagued the field for decades. One intriguing hypothesis is that SMN may have unique and essential functions in motor neurons, potentially beyond its classical role in the nucleus. Evidence supporting this hypothesis emerged when SMN protein was identified throughout neuronal axons and growth cones, where it exhibited fast bidirectional motility (Fan and Simard, 2002; Pagliardini et al., 2000; Tizzano et al., 1998; Zhang et al., 2003). Additionally, knockdown of SMN in a number of neuronal cell lines and in zebrafish resulted in decreased neurite outgrowth and axon branching abnormalities, raising the possibility that SMN somehow regulates the functions of the neuronal cytoskeleton underlying neurite outgrowth and guidance (Bowerman et al., 2007; McWhorter et al., 2003; Oprea et al., 2008; Tadesse et al., 2008; van Bergeijk et al., 2007).

Studies by two independent groups revealed a potential link between SMN and the neuronal cytoskeleton when it was discovered that SMN interacts with at least two proteins known to bind the zipcode sequence of β-actin mRNA: KSRP, the human homolog of ZBP2 (Tadesse et al., 2008), and hnRNP-R (Rossoll et al., 2002, 2003). Because SMN undergoes bidirectional axonal transport, a tantalizing hypothesis is that SMN functions within a ribonucleoprotein complex similar to ZBP1 and facilitates the shuttling of β-actin mRNA to motor neuron axons and growth cones. Data supporting this hypothesis emerged when cultured primary motor neurons from an SMA mouse model showed decreased β-actin protein in the growth cone which correlated with deficits in axon outgrowth, growth cone size, and β-actin mRNA localization (Rossoll et al., 2003). Yet, evidence supporting a critical role for SMN-mediated β-actin localization in SMA mouse models in vivo has been rare.

While there is one report of motor axons "overshooting" the motor endplate in the diaphragm muscle of an SMA mouse model (Kariya et al., 2009), most studies have not found any motor axon guidance defects in multiple SMA mouse models (Kariya et al., 2008; McGovern et al., 2008; Murray et al., 2010). Recent reports suggest instead that motor axon growth and guidance is normal in SMA mouse models, but that neuromuscular junction (NMJ) development or function is impaired. Further characterization of these mouse models has revealed NMJs with an abnormal, simplified morphology, and impaired synaptic vesicle release from motor axon nerve terminals (Cifuentes-Diaz et al., 2002; Kariya et al., 2008; Kong et al., 2009; McGovern et al., 2008; Murray et al., 2008). Because NMJ development

depends on bidirectional interactions between the motor axon and muscle motor endplate (Sanes and Lichtman, 1999), impaired or abnormal interactions between these cells could be the primary mechanism of pathogenesis. Interestingly, voltage-gated calcium channels, which function in axon guidance and neurotransmitter vesicle release, were also found to be mislocalized similar to β-actin in cultured SMA motor neurons (Jablonka et al., 2007). Thus, β-actin may still play a contributing factor in the pathogenesis of SMA but perhaps not in early motor axon development or guidance.

We hypothesized that the mislocalization of β-actin in SMA motor neurons leads to a loss-of-function of β-actin and motor neuron disease. In order to test this hypothesis, we generated a motor-neuron-specific β-actin KO mouse (MNs-*Actb*KO) by crossing a floxed β-actin allele to the motor-neuron-specific transgenic Cre line Mnx1 (also known as Hb9). Cre expression in this transgenic line is initiated at E9.5 corresponding with the onset of motor neuron specification. Surprisingly, MNs-*Actb*KO mice were viable and morphologically indistinguishable from controls (Cheever et al., 2011). MNs-*Actb*KO mice had normal numbers of motor neurons at both 6 and 12 months of age, and no indication of morphological NMJ abnormalities. This was corroborated by a number of behavioral and histological assays, which revealed normal motor function and a lack of any neurogenic atrophy in muscle histology. CNS-*Actb*KO mice, which also express Cre recombinase in motor neurons in addition to neurons and glia throughout the brain, were also subjected to the same motor function tests with comparable results (Cheever et al., 2012).

The study described above suggests that β-actin has a nonessential role in motor axon function; however, one caveat to this study is that it remains possible preexisting β-actin mRNA and protein were sufficient for the normal motor axon development that occurs rapidly in vivo. In order to more directly test whether β-actin is required for motor axon elongation as predicted by the cell culture work described above, we performed a peripheral nerve regeneration model in adult animals at a timepoint well after Cre-mediated recombination of the β-actin locus. Additionally, β-actin protein and RNA localization have also been directly implicated in the regenerative response in several different adult nerve regeneration paradigms, providing even further precedent for a critical role of β-actin in nerve regeneration (Lund and McQuarrie, 1996; Willis et al., 2005, 2011; Zheng et al., 2001). Here again, however, we found no significant differences in the functional or histological regeneration of motor axons lacking β-actin compared to controls (Cheever et al., 2011). Thus, β-actin does not appear to be required for motor axon

regeneration in vivo. It is likely that γ-actin or another actin isoform may compensate for the loss of β-actin function, or that the role of actin in general may be limited during peripheral nerve regeneration, where the activity of microtubules, for example, may be sufficient for successful regeneration.

While the studies described above indicate that β-actin is not required for motor neuron function or axonal regeneration, it is important to note that they do not conclusively rule out a role for β-actin in SMA. It is possible that the mislocalization of β-actin RNA and protein in SMA motor neurons leads to a gain-of-function rather than a loss-of-function mechanism, perhaps leading to the ectopic sequestering of accessory proteins or factors leading to motor neuron dysfunction and disease. Alternatively, some compensatory mechanism occurring in MNs-*Actb*KO mice may not also occur in SMA motor neurons, which would be an interesting and potentially therapeutically relevant discovery in itself. Although recent studies have continued to define axonal transport and mRNA localization roles for SMN in motor neurons (Fallini et al., 2011), it is important to note that evidence is also mounting in support of alternative pathogenic mechanisms in SMA involving tissue-specific disruptions in snRNP assembly and RNA splicing more aligned with the classical roles described for SMN (Gabanella et al., 2007; Workman et al., 2009; Zhang et al., 2008).

6.3.2. Neurological Effects of Mutations in Human β- and γ-Actin

Perhaps the most direct evidence for cytoplasmic actin functions in neuronal development and function in vivo comes directly from human patients. Seventeen distinct dominant missense mutations (five in *Actb* and 12 in *Actg1*) in the cytoplasmic actins have been identified as causative in various human diseases (Nunoi et al., 1999; Procaccio et al., 2006; Rendtorff et al., 2006; Riviere et al., 2012; van Wijk et al., 2003; Zhu et al., 2003). This includes one patient with an E364K mutation in *Actb* that resulted in mental retardation and significant immunodeficiency (Nunoi et al., 1999), while a set of monozygotic twins with an R183W mutation in the *Actb* gene exhibited multiple phenotypes including moderate cognition deficits, generalized dystonia, and cofilin–actin aggregates within the brain (Gearing et al., 2002; Procaccio et al., 2006). Western blotting of tissue or cells derived from these patients did not reveal any substantial decrease in β-actin expression, however, suggesting that these mutations cause disease either by a gain-of-function or dominant negative mechanism.

Six mutations in *Actg1* have been identified as the cause of the inherited genetic disorder DFNA20, which results in late onset hearing loss

(Rendtorff et al., 2006; van Wijk et al., 2003; Zhu et al., 2003). Although there is a significant neurological component to the proper reception and interpretation of sound, two pieces of evidence suggest that these mutations may not specifically perturb neuronal function. The first is that *Actg1* null mice have been generated and fully characterized (Belyantseva et al., 2009). No neuronal or neurobehavioral phenotypes have been identified to date, suggesting that γ-actin is not essential for neuronal development or function potentially due to overlapping functions with β-actin. The primary pathological finding from *Actg1* KO mice is the progressive loss of hair cell stereocilia (Belyantseva et al., 2009; Perrin et al., 2010), likely explaining the progressive hearing loss experienced by these mice and the human patients. Secondly, the hearing loss in human patients is nonsyndromic, indicating that hearing loss is the only symptom reported and suggesting that gross neuronal function is unperturbed.

One recent study, however, has identified *Actg1* mutations in human patients with neurological phenotypes, as well as three other distinct mutations in *Actb* that cause the same genetic disorder, Baraitser–Winter Syndrome (Riviere et al., 2012). In addition to nonneurological phenotypes including facial abnormalities, patients with Baraitser–Winter syndrome also exhibit lissencephaly, intellectual disabilities, and seizures suggesting a strong neurological component to this disease. Similar to what was reported for the two β-actin mutations described above, protein levels of the mutated actin isoforms were not perturbed in cultured lymphoblastoid cell lines derived from patients. Thus, here again missense mutations in the cytoplasmic actins likely cause disease by a gain-of-function or dominant negative mechanism. It is tempting to conclude that since mutations in both β- and γ-actin cause the same genetic disease that both genes have redundant functions. However, because of the likely gain-of-function nature of these mutations, it is difficult to discern the functions of the normal protein from those processes perturbed following gain-of-function mutations. Nevertheless, this new study demonstrates that proper regulation and function of the cytoplasmic actins is directly relevant to neurological function in humans.

7. FUTURE DIRECTIONS

For the first time, validated isoform-specific antibodies, biochemically relevant amounts of nearly pure actin isoform protein, and KO cell culture and animal models are available for the study of actin isoforms in neurobiology. Given how little is currently understood, and considering the

tremendous potential within areas yet to be explored, it is truly an exciting time for the study of actin isoforms in neuronal development and function. In this section, we will outline what we believe to be some of the critical short- and long-term experiments that will be needed in order to advance our understanding of the role of actin isoforms in neurons and the nervous system. The experiments outlined here complement the more specific experiments proposed in the preceding sections.

7.1. Molecular and Cellular Studies

Perhaps the most obvious and pressing question that remains to be answered is whether β-actin is specifically required for growth cone turning. The most direct way to answer this question is with an in vitro growth cone turning assay to eliminate potential confounding factors in more complicated systems. Until recently, this type of experiment was only routinely possible in cultured *Xenopus* neuronal systems. However, Welshhans et al. recently demonstrated that growth cone turning assays could be reliably performed using primary mouse cortical neurons in a neuron "ball" culture system (Welshhans and Bassell, 2011). Utilizing the same experimental setup with neurons cultured from a β-actin KO mouse (Cheever et al., 2012) could provide direct evidence as to whether β-actin is required at all for growth cone turning in a mammalian system. Interpretation of a positive result is relatively straightforward, but a negative result could also be due to genetic reprogramming of β-actin null neurons. The use of a recently described method to monitor spatiotemporal changes in G-actin concentration (Kiuchi et al., 2011) is one technique that could be used to ascertain whether transcriptional programs may be altered as seen in β-actin KO fibroblasts.

Cultures of β-actin null neurons could also be allowed to mature where dendritic spine formation, dynamics, and function could be directly assessed. In particular, miniature excitatory postsynaptic currents (mESPCs) could be measured in cultures of β-actin null neurons and compared to controls in order to gain insight on synaptic roles for β-actin. With the proper controls, any reduction in the amplitude of the mESPCs in β-actin KO neurons would suggest that dendritic spine and postsynaptic function is impaired. Positive results could then be followed up by staining for neurotransmitter receptors given that postsynaptic actin has been implicated in the regulation of the surface levels and localization of these receptors. The same experiments could also be performed with γ-actin null neurons in order to determine whether there are any isoform-specific roles in the regulation of dendritic spine function. These experiments promise to shed significant

light on whether synaptic transmission defects may be contributing to the behavioral defects observed in CNS-*Actb*KO mice.

We have also stressed the potential role of Ca^{2+} signals in promoting distinct functions for the cytoplasmic actin isoforms in neuronal development and function. Given that local Ca^{2+} signals factor into nearly every major function of neurons, and that actin in the Ca^{2+}-bound form is the only paradigm where functional distinctions between β- and γ-actin have been directly demonstrated, we feel that this issue merits particular attention. Until a method can be devised to "lock" a particular actin isoform in a Ca^{2+}- or Mg^{2+}-bound form, one experiment might be to knock β-actin-coding sequences into the γ-actin locus and vice versa. By utilizing this approach, Ca^{2+}-dependent responses to guidance cues and synaptic stimuli could be assessed without having to perturb global cytosolic Ca^{2+} signals, which undoubtedly interact with a large number of proteins besides actin. One would thus be able to determine whether a neuron expressing only γ-actin (but under the control of *Actb* and *Actg1* genetic loci) exhibits impaired morphological or functional responses to stimuli. If so, this might suggest that having β-actin with its distinct polymerization rate in the presence of Ca^{2+} may be functionally relevant. However, this interpretation is working under the assumption that the four amino acid difference between β- and γ-actin only affects the polymerization dynamics in the presence of Ca^{2+}, which may be an oversimplification.

7.2. In Vivo Studies

One commonality between our studies and those of other groups is that perturbation of β-actin localization or levels in general rarely affects the basal morphology of cultured neurons and many neurons in vivo for that matter. These findings may have been initially surprising, but now appear to suggest that β-actin may only be critical when a cellular response is stimulated. While these conditions can sometimes be mimicked in vitro, it is likely that in vivo experiments will be required particularly because there are essentially no phenotypes currently known in cultured β-actin null neurons to attempt to rescue. Although in vivo experiments can be much more time consuming, it is promising that many useful transgenic mouse lines are already available.

In our opinion, the most pressing in vivo question is whether any of the phenotypes observed in the CNS-*Actb*KO mouse model are due specifically to the inability of neurons to locally translate β-actin. The most straightforward way to address this question would be to generate a transgenic mouse expressing β-actin but lacking the 3′ UTR zipcode sequence, and determine

whether this mouse line could rescue the phenotypes observed in CNS-*Actb*KO mice. An even better experiment would be to specifically ablate the zipcode sequence at the endogenous *Actb* locus, but this is a far more time consuming and technically challenging endeavor. If either of these mice could rescue the phenotypes of the CNS-*Actb*KO mouse line, however, it would also be interesting to determine whether localization of specifically β-actin is required or the localization of any actin would suffice. Transgenic mice expressing γ-actin with the β-actin zipcode sequence could be generated and again crossed to the CNS-*Actb*KO line to determine whether this mouse could also rescue. Collectively, these experiments would establish whether the localization and local translation of β-actin is indeed required for proper neuronal development and function, and also whether the subtle amino acid differences between β- and γ-actin are functionally relevant in vivo. As described above, these experiments would ideally be conducted using a knock-in approach as opposed to randomly inserting transgenic lines, in order to preserve as much as possible the known and unknown regulatory elements at the endogenous loci.

Finally, resolving spatially and temporally the specific processes and cell types involved in the phenotypes of CNS-*Actb*KO mice could provide significant insight into the underlying molecular mechanisms occurring, and thus, the specific roles for β-actin. For example, one outstanding question is whether the behavioral phenotypes observed in the CNS-*Actb*KO mice are due to developmental morphological abnormalities, impaired synaptic transmission and plasticity in adults, or a combination of the two? A relatively straightforward way to address this question would be to utilize an inducible β-actin KO mouse model to ablate β-actin in adults, well after the morphogenesis of the brain has been completed. Upon verifying that the brain is in fact histologically normal, these conditional β-actin KO mice could then be analyzed using the same behavioral paradigms utilized for the CNS-*Actb*KO mice. Fortunately, inducible KO lines are already available for both β- and γ-actin (Zhang et al., 2012). If the primary pathological mechanism is indeed localized to synaptic transmission and plasticity, and not global morphological defects, then the inducible adult KO mice should present with a similar phenotype to the CNS-specific KO. There are also several hippocampal and cerebellar-specific Cre lines, including even some cell-type-specific lines, which could be used to further hone in on the cell populations responsible for the phenotypes observed in CNS-*Actb*KO animals (Dragatsis and Zeitlin, 2000; Goebbels et al., 2006; Tsujita et al., 1999; Zhang et al., 2004).

8. CONCLUDING REMARKS

In this chapter, we have presented evidence that distinct functions of actin isoforms likely comprise a previously unappreciated mechanism to regulate actin dynamics in neuronal development and function. While the localization and local translation of β-actin specifically has been the most investigated area of actin isoforms in neurons, the functional relevance of this phenomenon in vivo is unclear. Recent studies have revealed, however, that β- and γ-actin bound to Ca^{2+} exhibit significantly different polymerization kinetics. We propose that given the pervasiveness of Ca^{2+} signaling in neuronal function, that the distinct dynamics of Ca^{2+}-β-actin could be a critical player in mediating the localized actin polymerization required for cellular constriction events mediating tissue bending, synaptic plasticity, and behavior. Interestingly, ablation of β-actin function in the brain results in surprisingly restricted phenotypes, revealing novel and unanticipated roles for β-actin in the morphogenesis of select brain regions and regulation of specific behaviors. The contributions of γ-actin to neuronal development and function have only just begun to be characterized, with the distinct and overlapping functions of these isoforms in neurons remaining to be fully elucidated. Given the significant roles already assigned to actin isoforms in neuronal development and function, however, it is likely that even more exciting discoveries remain to be uncovered.

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CHAPTER FIVE

Endoplasmic Reticulum and the Unfolded Protein Response: Dynamics and Metabolic Integration

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Abstract

The endoplasmic reticulum (ER) is a dynamic intracellular organelle with multiple functions essential for cellular homeostasis, development, and stress responsiveness. In response to cellular stress, a well-established signaling cascade, the unfolded protein response (UPR), is activated. This intricate mechanism is an important means of re-establishing cellular homeostasis and alleviating the inciting stress. Now, emerging evidence has demonstrated that the UPR influences cellular metabolism through diverse mechanisms, including calcium and lipid transfer, raising the prospect of involvement of these processes in the pathogenesis of disease, including neurodegeneration, cancer, diabetes mellitus and cardiovascular disease. Here, we review the distinct functions of the ER and UPR from a metabolic point of view, highlighting their association with prevalent pathologies.

1. INTRODUCTION

Our understanding of cellular reticulum began in 1945 when it was first described as a "lace-like" structure in the ground substance of cultured cells and examined *in toto* by electron microscopy. The term "endoplasmic reticulum" (ER) was coined in 1952 by Porter & Kallman to describe

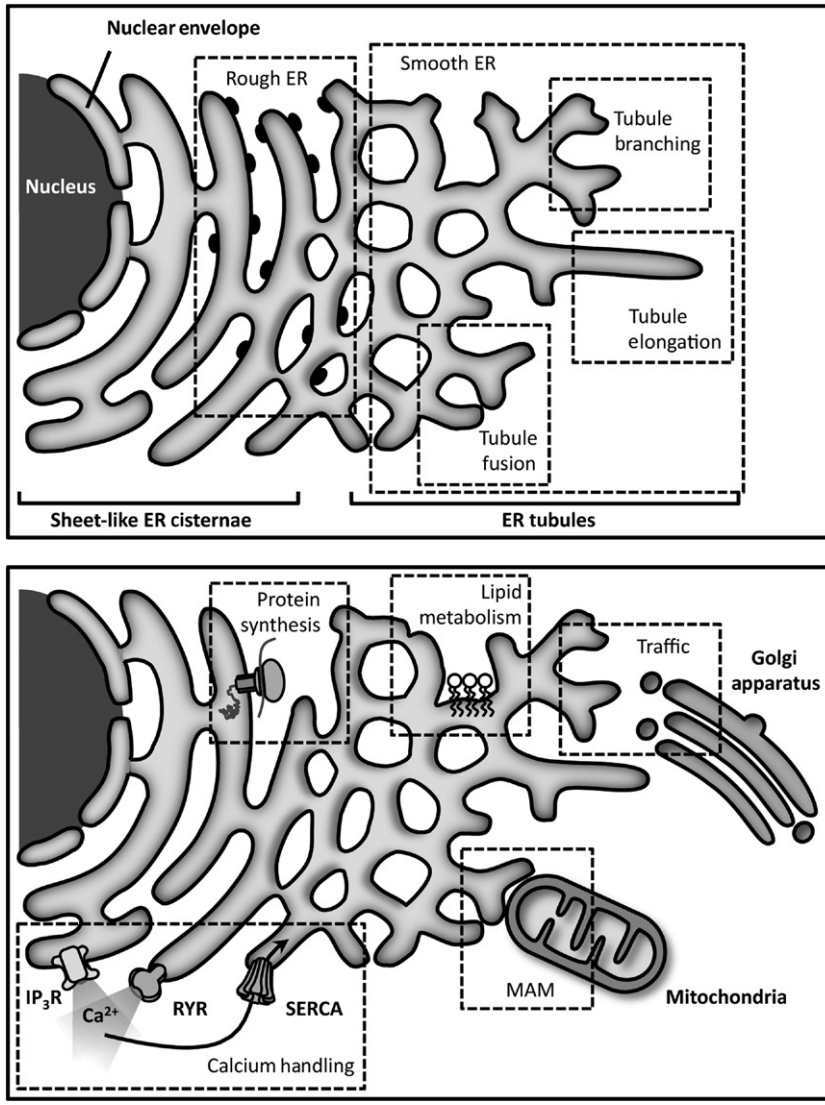

Figure 5.1 ***ER structure and general functions***. Upper panel: The ER can be subdivided into three well-defined domains, the sheet-like ER, the tubular ER and the nuclear envelope. The first one is characterized as being rich in ribosomes, for which it received the name rough ER. Since the tubular ER contains fewer ribosomes, it is commonly called smooth ER. Both the sheet-like and tubular ER are highly dynamic and interconvert between each other constantly. Most studies suggest that the tubular ER has the ability to fuse, elongate and branch dynamically inside the cell. Lower panel: The ER fulfills diverse functions in the cell, like calcium homeostasis through the use of a series of channels, pumps and buffer proteins. It is essential for lipid and protein synthesis, as well as the quality control and degradation of proteins. Together with the Golgi apparatus, it takes part in the process of cell trafficking, which is important for the export of products from reticulum toward the outside of the cell. Finally, the ER also regulates the function of other organelles, such as mitochondria through dynamic interaction zones called MAM.

the observation of the preferential localization of vesicular elements in the perinuclear region of the cytoplasm, known as *endoplasm* (Palade, 1956). Early studies described two major types of membranous structures of ER, distinguishable by their biochemical and morphologic properties and their sedimentation features. One type corresponds to the tubular or "tadpole-like" structure recovered in the low-density fraction, and the other to the spherical vesicles present in the high-density fraction (Heuson-Stiennon et al., 1972).

The ER, although frequently associated with the cellular exo-endocytic pathway, is a complex organelle in terms of both its structure and function (Fig. 5.1). It plays critical roles in a wide range of processes, including (a) synthesis, folding, modification, and transport of proteins; (b) synthesis and distribution of phospholipids and steroids; (c) storage of calcium ions within its lumen and their regulated release into the cytoplasm (Schröder, 2008). Perturbations in any of these functions results in ER stress and aggregation of misfolded proteins. ER stress has been observed during physiological conditions, such as nutrient deprivation and the differentiation of type B lymphocytes into plasma cells, as well as in pathological conditions, such as viral infection, ischemia/reperfusion and cardiomyocyte hypertrophy.

2. STRUCTURE, FUNCTION AND DYNAMICS OF ENDOPLASMIC RETICULUM

2.1. General Structure of Endoplasmic Reticulum

2.1.1. Rough ER Sheets and Smooth ER Tubules

A number of approaches have established that the ER is a continuous compartment extending from nucleus to cytosol. Based on its structure, the ER is classically subdivided in the ribosome-studded rough endoplasmic reticulum (RER) and the ribosome-free smooth endoplasmic reticulum (SER) (English et al., 2009). Cells that secrete large amounts of protein are rich in RER, while steroid-synthesizing and muscle cells have abundant SER. In many cells, RER and SER do not occupy spatially segregated regions; however, in some cells such as hepatocytes and neurons, the smooth and rough portions of the ER occupy different cellular areas (Borgese et al., 2006). The SER morphology differs from that of RER by its typically more complex, tubular network and greater numbers of branch points. Xenobiotic-metabolizing enzymes are also preferentially located in the SER (Orrenius and Ericsson, 1966).

A more contemporaneous classification divides the ER in three domains: the nuclear envelope (NE), the sheet-like cisternae, and the polygonal array of tubules (Shibata et al., 2006). The NE controls the flow of information between the cytoplasm and the nucleoplasm, and consists of a double membrane enclosing a lumen. The NE surrounds the nucleus, with the inner and outer membranes connected only at the nuclear pores, the former serving as a scaffold for chromatin organization localized at the inner membrane (Dreier and Rapoport, 2000).

In yeast, there are more ribosomes in the cisternae than in the tubules, suggesting that either cisternae are better suited for ribosome binding and/or ribosome binding stabilizes cisternal ER structure. In animal cells, the protein translocation machinery is also enriched in this structure. For example, the expression of p180, a yeast ribosome-binding protein anchored to the ER membrane, increases secretory activity. Moreover, ectopic expression of p180 in nonsecretory mammalian cells induces biogenesis of RER membrane, while upon p180 knockdown, THP-1 secretory cells decrease biogenesis in the area occupied by RER (Benyamini et al., 2009).

Reticular networks can be formed de novo in *Xenopus* egg extracts studied in vitro, in a cytoskeleton-independent manner but dependent on energy and protein elements which drive microsome fusion into tubule formation (Dreier and Rapoport, 2000). The integral membrane protein Reticulon 4a (Rtn4a), a member of an ER membrane-localized protein family, has been implicated in tubule formation. Consistent with this role, Rtn4a is absent in sheets and NE, but enriched in ER tubules (Voeltz et al., 2006). The yeast protein, Yop1p, and its mammalian ortholog, DP1, comprise another family of integral membrane proteins that form oligomers on the tubular ER and are fundamental for tubule formation (Shibata et al., 2008). Rtn4a expression generates more tubules, while Rtn4a depletion along with DP1/Yop1 converts the peripheral ER into sheets (Voeltz et al., 2006).

In muscle cells, a variant of the ER, termed sarcoplasmic reticulum (SR) is present, whose main function is to control calcium release for muscle contraction. In striated muscle, the plasma membrane forms long invaginations, called transverse tubules (T-tubules) that penetrate into the cytoplasm. These structures insert between two terminal SR cisternae, forming a triad. This highly organized T-tubule system is essential for rapid and precise excitation–contraction coupling. Different proteins collaborate in T-tubule biogenesis, and in the formation and maintenance of the triad in both cardiac and skeletal muscles: caveolae are cell surface structures that initiate

membrane invaginations, while the invagination and tubulation processes are possible due to the participation of caveolin 3, amphiphysin 2 and dysferlin among others. In addition, a number of proteins play important roles in stabilizing the triad structure in skeletal muscle, including mitsugumins, junctophilins, myotubularin, ryanodine receptor (RyR), and the dihydropyridine receptor (Cav1.1 calcium channel) (Al-Qusairi and Laporte, 2011).

2.1.2. Nuclear Envelope

The NE is a highly specialized and selectively permeable double membrane that surrounds the genetic material of the cell. Electron microscopic analysis revealed early on that NE and ER form a continuous structure (Watson, 1955), sharing some common components, while others, like nuclear pore-forming nucleoporins (NUPs), are specific for the NE. Other important components are the transmembrane proteins of the NE, which interact with laminin, a protein localized in the internal surface of the NE, and with chromatin, thereby participating in anchorage of the genetic material and in gene regulation. On the other hand, KASH domain-containing proteins are small transmembrane proteins at the external face of the NE that associate with cytoskeletal proteins, thus determining the shape and positioning of the NE (Hetzer, 2010).

The NE is a highly dynamic structure, which is modulated during different stages of the cell cycle. In stage G2, the nucleus must duplicate its size by not only increasing its volume, but also by duplicating its NE proteins; during mitosis, specifically in prophase, the NE disintegrates. The processes that lead to dismantling of the NE are not fully understood, however, these events are preceded by loss of NUPs and transfer of the NE proteins to the mitotic ER. Following anaphase, NE restructuring occurs and terminates in complete morphological and functional restructuring of the nucleus. Our understanding of the NE restoration process is controversial, as there are two leading theories. The first suggests that the NE is fragmented into small vesicles that are not degraded completely during mitosis and subsequently merge to restructure the NE. The second theory proposes that the ER differentiates into the NE, which is supported by the mitotic ER structure and enriched in NE proteins (Hetzer, 2010).

2.1.3. Lipid-Raft-like Domains

Lipid rafts are cholesterol-rich plasma membrane domains which may contain caveolin. Lipid-raft-like domains are defined as caveolin-free plasma membrane regions enriched in cholesterol and members of the prohibitin

domain-containing protein family, such as KE04p and C8orf2. These proteins are essential for the maintenance of lipid-raft-like structures. Although it is known that the ER has lower levels of cholesterol and glycosphingolipids than the plasma membrane and other organelles, two proteins homologous to KE04p and C8orf2, Erlin-1 and Erlin-2 (for ER lipid-raft protein), are found in the ER, suggesting the existence of lipid-raft-like domains in the ER (Browman et al., 2006).

Another protein involved in the maintenance of lipid-raft-like domains is the sigma-1 receptor chaperone (Sig-1R), a cholesterol-binding protein (Hayashi and Su, 2010). Sig-1R is also involved in mitochondria–ER binding through microdomains enriched in cholesterol and ceramides (Hayashi and Fujimoto, 2010), which form part of the mitochondria-associated ER membrane (MAM) structure.

2.2. Functions of Endoplasmic Reticulum

2.2.1. Protein Synthesis in Endoplasmic Reticulum

Proteins targeted to the ER and other organelles or destined for secretion must be incorporated into the ER. Nascent polypeptides possess a signal sequence in their N-termini that targets them to the ER. This sequence is not conserved and generally contains hydrophobic amino acids in the core region. This sequence is recognized by the signal recognition particle (SRP), consisting of six polypeptides bound to a small RNA, which cycles between the cytoplasm and the ER membrane. When the SRP recognizes and binds to the signal sequence of nascent polypeptides, a pause in translation occurs. Then, the SRP–ribosome complex translocates from the cytoplasm to the SER membrane and binds to the SRP Receptor (SR) (Corsi and Schekman, 1996). Once this process is complete, the SRP and SR are released, and the nascent polypeptide is translocated by the translocon complex (TC) formed by more than 25 polypeptides (Nikonov and Kreibich, 2003). The core of the TC is Sec61, a protein required for the translocation of both secretory and membrane proteins. In mammals, Sec61 is a complex formed by three subunits: Sec61α, Sec61β and Sec61γ. This complex possesses ribosomal binding sites, and its structure forms a pore through which the polypeptide traverses the membrane. The translocation-associated membrane protein (TRAM), another integral membrane protein component of the TC is required for the translocation of some precursors, like ppαF. The above-mentioned process is known as cotranslational translocation; however, targeting to the ER can also occur posttranslationally. This event occurs independently of SRP in both yeast and mammalian cells. In

yeast, Sec62p, Sec63p, Sec71p and Sec72p form a tetrameric protein complex that may function as a receptor in this type of translocation process. Several chaperones are known to participate, including Sec61 (Wilkinson et al., 1997).

Another important part of protein synthesis is folding and proper assembly of the nascent polypeptide. To accomplish this, the ER contains a variety of proteins that assist in the process. Protein disulfide isomerase (PDI) and ERp57, for example, are thio-oxidoreductases that catalyze disulfide bond formation by means of the oxidative capacity provided by ER oxidoreduction 1 (ERO1). Glucose-regulated protein 78 (BiP or GRP78), a chaperone of the heat-shock protein family, recognizes and stabilizes unfolded proteins, and participates in posttranslational translocation (Ni and Lee, 2007). Another set of proteins is responsible for folding quality control, a process that will be described later in the text.

2.2.2. Endoplasmic Reticulum and Calcium Homeostasis

Calcium is recognized as one of the most important second messengers in the cell; it participates in a wide variety of cellular processes, including protein synthesis, muscle contraction, gene expression, secretion, cell cycle, metabolism and apoptosis (Coe and Michalak, 2009). Given its diverse functions, intracellular free calcium concentrations are tightly regulated. In this respect, a series of buffer proteins, pumps and carriers of calcium participate in this process, serving to diminish or increase calcium concentrations according to cell requirements.

The ER is the principal organelle involved in calcium homeostasis, and in turn, many of its diverse functions, like protein folding and glycosylation, are calcium-dependent by virtue of the fact that the enzymes involved are calcium-dependent (Kuznetsov et al., 1992). Cytoplasmic calcium regulation depends on the activity of a series of transporters in the ER membrane. Sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) is a calcium pump that imports calcium from the cytoplasm into the ER lumen, thereby maintaining low cytoplasmic calcium levels (Strehler and Treiman, 2004). On the other hand, the RyR and the inositol 1,4,5-trisphosphate receptor (IP3R) are calcium channels that release this ion back into the cytoplasm (Marks, 2001; Zalk et al., 2007; Taylor and Tovey, 2010).

2.2.2.1. Sarcoplasmic/Endoplasmic Reticulum Calcium-ATPase

SERCA is a type P ATPase pump that transports two calcium ions in exchange of the hydrolysis of one ATP molecule, functioning against a

calcium gradient to restore luminal ER calcium levels (Guerrero-Hernandez et al., 2010). Small proteins, such as phospholamban and sarcolipin, modulate SERCA activity according to cellular requirements and/or extracellular signals (Periasamy and Kalyanasundaram, 2007; Brini and Carafoli, 2009; Guerrero-Hernandez et al., 2010).

Distinct genes code for the three different pumps, SERCA 1, 2 and 3, generating a total of 10 isoforms by alternative splicing. SERCA1 is expressed principally in skeletal muscle, with two known isoforms, SERCA1a (adult) and SERCA1b (fetal). SERCA2a is mainly expressed in cardiac and skeletal muscle, while SERCA2b and SERCA3 are present in nonmuscle cells, the latter being the most ubiquitous of the isoforms (Periasamy and Kalyanasundaram, 2007; Brini and Carafoli, 2009).

2.2.2.2. Ryanodine Receptor

RyR is an ER and SR calcium channel important for regulation of calcium transients in excitable cells. Three isoforms of this receptor are known to exist, RyR1, 2 and 3; RyR1 is mainly present in skeletal muscle, RyR2 in cardiac muscle, and RyR3 in nerve fibers and in nonexcitable cells (Marks, 2001; Zalk et al., 2007).

RyR conductance is governed by several proteins, such as voltage-gated channels (Cav 1.1/Cav 1.2 or dihydropyridine receptor), protein kinases (PKA and CaMKII), calcium-binding proteins (Calmodulin and Calsequestrin) and the FKBP-12 and 12.6 proteins (Lanner et al., 2010). Also, divalent cations, such as calcium and magnesium, and nucleotides, such as ATP, are other important regulators of RyR (Lanner et al., 2010). In addition, RyRs possess a series of sulfhydryl and cysteine groups sensitive to the redox state of the cell (Sun et al., 2008; Donoso et al., 2011).

2.2.2.3. IP3R

The IP3R is a tetrameric protein that transports calcium from the ER into the cytoplasm. It is responsible for calcium transients in nonexcitable cells, nuclear calcium regulation and calcium transfer between ER and mitochondria (Taylor and Tovey, 2010).

The IP3R requires elevated calcium levels to be activated by its agonist, IP3. IP3 binds to the IP3R at four sites in a cooperative manner. In parallel, calcium regulates IP3 function through calcium-binding sites, which are different from those used for ion transport. Depending on the calcium concentration, IP3Rs possess a two-phase response. Low amounts of calcium increase the IP3R response, independent of IP3 levels. On the contrary,

high calcium concentrations have inhibitory effects on the channel (Taylor and Tovey, 2010). Furthermore, the IP3R is modulated by covalent modifications, such as phosphorylation of key residues by PKA, ERK and/or PKC (Patterson et al., 2004). Nucleotides, such as ATP and NADH, also play an important role in channel modulation, linking the energetic needs of the cell to IP3R activity (Patterson et al., 2004; Taylor and Tovey, 2010).

Finally, apoptotic proteins like caspase-3, calpains, and cytochrome c interact with and regulate IP3Rs, thereby contributing to life or death decisions of the cell (Patterson et al., 2004; Taylor et al., 2004).

2.2.2.4. Calcium Buffering

Calcium-binding chaperones, such as calreticulin and calnexin, also play an important role in ER luminal calcium regulation. By sequestering free calcium, chaperones work as ion buffers. Owing to the efficient actions of these proteins, the free calcium concentration in the ER is only 50–500 μM, whereas the actual luminal calcium concentration ranges around 2 mM (Coe and Michalak, 2009).

Calreticulin, the glucose-regulated protein 94 (GRP94) and BiP/GRP78 are the most abundant calcium-binding chaperones in the ER. These proteins bind calcium with high affinity in their C-terminal domain where they possess several calcium-binding sites (Michalak et al., 2009). In the SR, the main chaperone responsible for calcium regulation is the calreticulin homolog, calsequestrin, which is of great importance in muscle contraction, not only because of its buffering capacity but also its ability to modulate RyR function (Michalak et al., 2009).

2.2.3. Lipid Synthesis at Endoplasmic Reticulum

Lipids fulfill several functions essential for cellular homeostasis. They are employed as a backup energy source, signaling molecules, and as membrane components, among other functions. Cellular lipids are quite heterogeneous, existing in the form of fatty acids, phospholipids, cholesterol, and sphingolipids (Laplante and Sabatini, 2009). The ER plays an essential role in lipid biogenesis, mainly in the synthesis of glycerophospholipids and sphingolipids, the major components of biological membranes. ER enzymes such as glycerol-3-phosphate acyltransferase-like and 1-acylglycerol-3-phosphate-*O*-acyltransferase transform glycerol and fatty acids into phospholipid precursors, such as triglycerides and diacylglycerol phosphate (DGP). In the ER lumen, DGP is dephosphorylated by phosphatidic acid phosphatases to form diacylglycerol (DG), which is converted to phosphatidylcholine and phosphatidylethanolamine.

Phosphatidylinositol is synthesized in the ER by phosphatidylinositol synthase, and derivatives with different levels of phosphorylation are formed in the ER and play an important role in signaling and vesicle trafficking (Fagone and Jackowski, 2009).

De novo synthesis of sphingolipids and ceramides begins in the cytoplasm. Then, intermediates are generated in the ER and the process concludes in the Golgi apparatus and nucleus. The coordinated action of serine palmitoyltransferase, 3-ketodihydrosphingosine reductase and dihydroceramide synthase in the ER, convert serine and palmitoyl-CoA into cell membrane components (Gault et al., 2010).

Cholesterol synthesized at the ER is important for cell function due to this molecule's structural role in membranes and as a precursor of various steroid hormones and bile salts. The 3-hydroxy-3-methyl-glutaryl CoA reductase, found in the membrane of the ER, participates in the first phase of cholesterol synthesis, which leads to the combination of three molecules of acetyl-CoA to form mevalonate. Mevalonate is then transformed into 3-isopentyl pyrophosphate. The condensation of six molecules of isopentyl pyrophosphate gives rise to squalene, owing to the action of a series of transferases and squalene synthase. Squalene is then cyclized by lanosterol cyclase, yielding lanosterol. After 19 reactions of bond reduction and methyl-group elimination, catalyzed by various enzymes, cholesterol is ultimately generated. Steroid hormones and bile acids are formed subsequently through the action of other pathways (Jo and Debose-Boyd, 2010; Maxfield and van Meer, 2010).

2.3. Endoplasmic Reticulum Dynamics

The tubular structure of the ER is highly dynamic and undergoes constant morphological remodeling. The cytoskeleton formed by microtubules (MTs), microfilaments and intermediate filaments plays a crucial role in the organization and structure of the ER. Treatment with depolymerizing agents reversibly and dramatically alters ER shape, causing slow retraction from the periphery to the center of the cell. MT and ER are highly interdependent structures. While the distribution of ER and MT are not identical, ER elongation and MT polymerization are tightly connected. The maintenance of the ER network requires the MT system (Terasaki et al., 1986). For example, the RER-specific membrane protein, CLIMP-63, mediates the interaction between this organelle and MTs through its cytosolic domain, and is responsible for the restraining mobility of the TC (Nikonov et al., 2007).

Two different mechanisms of ER tubule elongation along MT exist. The first, known as tip attachment complex (TAC), involves STIM1, a single-pass membrane protein mainly localized to the ER. This protein directly interacts with EB1, an MT-plus-end-binding protein (Grigoriev et al., 2008). In the TAC mechanism, the ER membrane selectively attaches to the growing end of an MT in regions where ER networks are dense and MT-plus ends are abundant. This mechanism allows ER tubule stretching dependent on MT growth. For the second, the Sliding mechanism, the tip of the ER tubules binds to an existing MT shaft, forming a sliding attachment that moves toward the plus end of a MT (Waterman-Storer and Salmon, 1998). This mechanism is faster and more prevalent than TAC, occurring mainly in MT harboring acetylated α-tubulin (Friedman et al., 2010).

ER tubules are capable of fusion and fission/branching processes, fundamental for the formation of the reticulated network (Anderson and Hetzer, 2007). It is known that ER fusion requires GTP and a family of proteins called *atlastins*, which possess GTPase activity and belong to the dynamin superfamily. In vertebrates, three isoforms are known, atlastin 1, 2, 3, and only one ortholog exists in invertebrates. Atlastins interact with different ER-shaping proteins, and these interactions are required for the formation of ER junctions (Barlowe, 2009; Farhan and Hauri, 2009; Hu et al., 2009). In *Drosophila*, depletion of atlastin produces ER fragmentation, while its overexpression favors ER fusion. In vitro, reconstitution of atlastin in liposomes promotes GTP-dependent fusion. Moreover, in humans, atlastins take part in the formation of the reticulated network and membrane remodeling (Hu et al., 2009; Muriel et al., 2009).

2.4. Contacts between ER and Other Organelles

Peripheral ER interacts with almost all cytoplasmic organelles. In this context, the ER forms physical contacts with mitochondria, with important functional implications. In yeast, this physical interaction is mediated by the ER–mitochondria encounter structure (ERMES), formed by four components: the mitochondrial outer-membrane proteins Mdm10 and Mdm34, the ER integral membrane protein Mmm1, and the cytosolic protein Mdm12 (Kornmann et al., 2009). In mammals, the contact sites are termed MAMs, and have a composition that differs from that of the membranes of other organelles. For one, the MAMs are enriched in cholesterol, which gives them a characteristic density and architecture (Hayashi and Fujimoto, 2010). Structural proteins that compose the MAM, include Mitofusin-2 (Mfn2), a GTPase protein present on the surface of both organelles that

connects the two by forming dimers (de Brito and Scorrano, 2008). Other components, such as the sorting protein PACS2 or the GTPase Rab32, regulate MAM composition according to cellular requirements (Myhill et al., 2008; Bui et al., 2010).

Given that the ER and mitochondria represent the cell's main source and sink of calcium, respectively (Berridge, 2002), it is not surprising that MAMs play a key role in calcium homeostasis (Hayashi et al., 2009). These interorganelle contacts allow the formation of microdomains of high calcium concentration at the surface of mitochondria, thereby facilitating rapid ion uptake (Rizzuto et al., 1998). Proteins known to be involved in calcium handling have been shown to be enriched in MAM. Examples here include IP3Rs (Mendes et al., 2005; Szabadkai et al., 2006), the RyR (García-Pérez et al., 2008; Kopach et al., 2008), the Sig-1R (Hayashi and Su, 2007), and perhaps even calnexin (Myhill et al., 2008), emphasizing the intimate relationship between ER and mitochondria in regulating calcium homeostasis (Mironov and Symonchuk, 2006). Conversely, calcium regulates relative positioning of ER and mitochondria via the autocrine motility factor receptor (AMFR) (Wang et al., 2000), apparently another MAM-enriched protein (Registre et al., 2004; Goetz and Nabi, 2006).

ER and mitochondrial dynamics are highly interrelated and their contacts are maintained in spite of this dynamism. Recent data obtained in yeast demonstrate that the contacts are conserved during mitochondrial fission. Moreover, fission itself can take place in the ER–mitochondria junctions (Friedman et al., 2011).

Contacts between plasma membrane and ER have been described in several cell types. The proteins STIM1 at the ER and ORAI1 at the plasma membrane regulate this interaction when calcium is depleted (Toulmay and Prinz, 2011). Another important group of proteins present in the ER–plasma membrane contacts is the Osh family. Osh3, for example, localizes to the contacts according to the level of PI4P. High PI4P levels recruit and activate Osh3 at contacts sites, leading to its interaction with VAP proteins in the ER and the activation of phosphatases, such as Sac1 (Stefan et al., 2011).

The ER and Golgi apparatus are functionally linked as constituents of the secretory pathway; however, direct physical contacts between the two organelles have been described, as well. Ceramides produced in the ER are transported to the *trans*-Golgi to be converted to sphingomyelin. In this case, appropriate trafficking requires the cytosolic protein CERT, which interacts with VAP on the ER surface (Kawano et al., 2006). Another example of

a direct mechanism and contact between both organelles is the nonvesicular transport of phosphatidylinositol and diacylglycerol, which also depends on VAP, and, in the case of phosphatidylinositol, requires the Nir2 protein (Peretti et al., 2008).

As noted above, regulation of Ca^{2+} by the ER is a key component of cellular signaling, adaptation and survival. Additionally, the ER has been implicated in a complex communication system with other organelles, including the Golgi apparatus, the plasma membrane, the nucleus, and mitochondria. The next sections of this review will focus on these communication processes, particularly, on the ability of ER Ca^{2+} signals to modulate cellular bioenergetics, thereby influencing cellular metabolism during stress and survival.

3. ER STRESS AND UNFOLDED PROTEIN RESPONSE

3.1. General Aspects of the UPR

Conditions that alter ER homeostasis and proper ER functioning generate a state known as *ER stress*. Many different factors may lead to this state, which can be very detrimental to cell integrity, due to accumulation of toxic, unfolded proteins within the ER lumen. For this reason, restoring ER homeostasis is essential for cell survival. Cells subject to ER stress activate a series of processes, which, if insufficient to alleviate the stress, lead to cell death.

The cellular response to ER stress is known as the unfolded protein response (UPR). In principle, the UPR seeks to restore the normal functioning of the ER, using multiple strategies acting in parallel and in series. For example, expression of ER chaperone proteins increases to prevent protein aggregation and facilitate correct protein folding. Also, the amount of protein in transit through the ER is reduced by temporary inhibition of protein translation. Furthermore, ER volume increases by stimulating the synthesis of membrane lipids. Finally, degradation of unfolded proteins increases by activating the process of endoplasmic reticulum-associated protein degradation (ERAD).

During UPR, perturbations in ER homeostasis are sensed and transduced to the cytoplasm and nucleus causing a compensatory response. Several ER stress sensors are involved, all of which harbor luminal, transmembrane and cytoplasmic domains. Any increase in the concentration of misfolded proteins is detected by the luminal sensor domain and then transduced to the cytoplasm and nucleus, through different signals (Fig. 5.2).

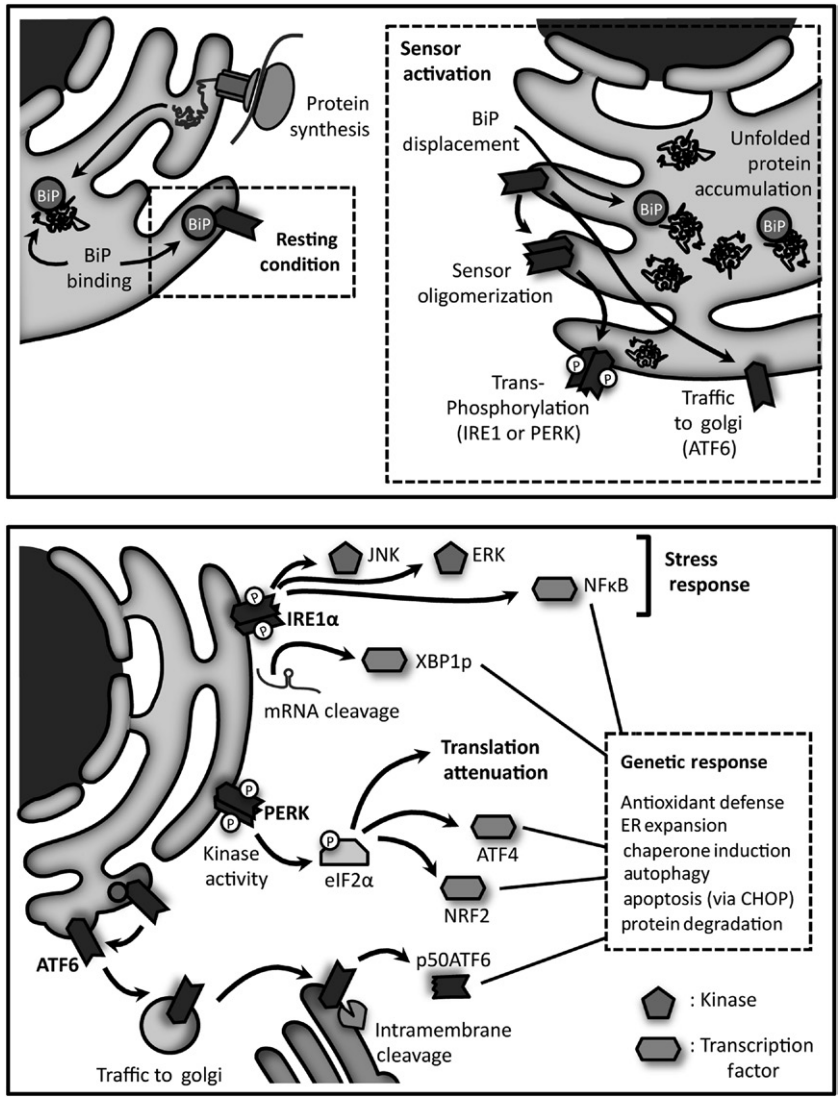

Figure 5.2 ***ER stress signaling***. Upper panel: In resting conditions, the stress sensors IRE1 and PERK and ATF6 interact with Bip/GRP78. Accumulation of misfolded proteins in the ER lumen separates the chaperone from each sensor. IRE1α and PERK activation involves oligomerization and transphosphorylation of their cytosolic effector region. ATF6 activation, on the other hand, requires its transport to the Golgi, where it is sequentially cleaved by S1P and S2P. A recent model for IRE1α activation, involves an initial dissociation of BiP/GRP78 that drives sensor oligomerization, while subsequent binding to unfolded proteins leads to activation. Lower panel: The signaling engaged by the three sensors activates several transcription factors (XBP1, ATF4, p50ATF6, NF-κB, CHOP) and protein kinases (JNK, AKT), leading to the establishment of a genetic program termed unfolded protein response. This adaptive response involves chaperone induction, activation of degradation pathways like the proteasome and autophagy, ER expansion and increased antioxidant defense. Also, the PERK pathway inhibits general mRNA translation, through eIF2α phosphorylation. If the cell does not reduce the misfolded protein overload, apoptosis is triggered.

Three branches of UPR have been described: PERK, IRE1α, and activating transcription factor (ATF6). In the rest of this chapter, we will review general aspects of the sensors and their relationship to various aspects of cell metabolism.

3.2. ER Stress Sensors

3.2.1. IRE1 Pathway

The inositol-requiring enzyme-1 (IRE1) is the most highly conserved branch of the UPR. It is an ER transmembrane protein that consists of an N-terminal luminal sensor domain, a single transmembrane domain and a C-terminal cytosolic effector region that manifests both kinase and endoribonuclease activity (Walter and Ron, 2011). The IRE1 pathway has been widely described by Peter Walter's group in *Saccharomyces cerevisiae*, who observed that it increases the expression of ER chaperones, thereby enhancing cell viability during ER stress (Cox et al., 1993). IRE1 senses unfolded proteins in the ER lumen, which induces receptor oligomerization and transphosphorylation within the cytosolic effector region (Shamu and Walter, 1996). The cytosolic domain also exhibits site-specific endoribonuclease activity that cleaves a small stem–loop structure from splice junctions in the mRNA of the nuclear factor Hac1 (Sidrauski and Walter, 1997; Gonzalez et al., 1999). Hac1 is a basic-leucine zipper (bZIP) transcription factor, which binds to the promoter of UPR-regulated genes and is more resistant to ubiquitin-dependent degradation when the IRE1-mediated splicing replaces its C-terminal (Cox and Walter, 1996).

Mammalian cells have two paralogs of IRE1, IRE1α and IRE1β, sharing structural similarity but different functions. Under ER stress conditions, IRE1α catalyzes the splicing of X-box-binding protein 1 (XBP1) mRNA, the mammalian ortholog of Hac1, while IRE1β mediates the site-specific cleavage of 28S rRNA and translational attenuation (Tirasophon et al., 1998; Iwawaki et al., 2001). XBP1 is not the only target of IRE1α. Surprisingly, IRE1α controls its own expression by cleaving its own mRNA (Tirasophon et al., 2000); another 13 mRNA candidates of IRE1α cleavage have recently been identified (Oikawa et al., 2010).

The capacity of IRE1α to sense ER stress depends on its dissociation from BiP/GRP78 and on its direct interaction with unfolded proteins. These two observations are incorporated into the two-step model of IRE1α regulation, where initial dissociation of BiP/GRP78 from IRE1α drives oligomerization, while subsequent binding to unfolded proteins leads to IRE1α activation (Kimata et al., 2007; Pincus et al., 2010). A recent study

suggests that IRE1α also senses changes in membrane composition. Mutant IRE1α incapable of sensing unfolded proteins is activated by depletion of the membrane lipid components and by deletion of genes involved in lipid homeostasis (Promlek et al., 2011). Indeed, IRE1 gene deletion confers auxotrophy for inositol, an important component of phospholipids in yeast cells (Nikawa and Yamashita, 1992).

Other proteins also control the activation of IRE1α. Indeed, the pro-apoptotic Bcl-2 family members Bax and Bak form a protein complex with the cytosolic domain of IRE1α, essential for its activation (Hetz et al., 2006). Bax inhibitor 1 (BI-1), an ER-resident protein, also forms a complex with IRE1α, decreasing its endoribonuclease activity (Lisbona et al., 2009; Madeo and Kroemer, 2009).

When IRE1α is activated, the luminal ER stress signal is transduced to the cytoplasm, activating various signaling pathways. One of these pathways is initiated by IRE1α-mediated splicing of XBP1 mRNA, which generates a protein that differs from its unspliced protein (XBP1u) in the C-terminal region (Yoshida et al., 2001). XBP1 is a potent transactivator that regulates genes involved in ER protein synthesis, folding, glycosylation, ERAD, redox metabolism, autophagy, lipid biogenesis and vesicular trafficking (Walter and Ron, 2011). XBP1u does not act directly as a transcription factor, but rather functions as negative feedback regulator of XBP1 by sequestering the protein from the nucleus and promoting its degradation by the proteasome (Yoshida et al., 2006). Moreover, a hydrophobic patch in the XBP1u nascent peptide chain recruits its mRNA (attached to the ribosome) to the ER membrane, where it is cleaved by IRE1α to produce pXBP1s (Yanagitani et al., 2009).

IRE1α also activates JNK by recruiting the TNF receptor-associated adapter protein TRAF2 (Urano et al., 2000). This activation by ER stress requires the presence of MAP3K, ASK1 and ASK1-interacting protein-1 (AIP1), a transducer in the ASK1-JNK signaling pathway (Nishitoh et al., 2002; Luo et al., 2008). JNK activation is associated with cell death (Kim et al., 2006), and also promotes survival through the activation of c-Jun (Zhao et al., 2008; Fuest et al., 2011), increased BiP/GRP78 and GRP94 expression (Shinkai et al., 2010), as well as activation of autophagy after ER stress (Ogata et al., 2006). Similar to JNK, ER stress induces activation of NF-κB, which is mediated by IRE1 and TRAF2 (Kaneko et al., 2003). This activation of NF-κB controls the expression of manganese superoxide dismutase (MnSOD), an antioxidant enzyme localized in the mitochondrial matrix (Kaneko et al., 2004).

Important modulators of cell metabolism also regulate the IRE1α pathway. It was recently described that both XBP1 splicing and JNK activation are controlled by the mTORC1 pathway, the major sensor of nutrient and energy availability in the cell (Ozcan et al., 2008; Pfaffenbach et al., 2010; Kato et al., 2012). IRE1α is phosphorylated by PKA, controlling glucagon-mediated expression of gluconeogenic genes (Mao et al., 2011). p85, a repressive regulatory subunit of PI3K, also interacts with XBP1, increasing its nuclear translocation and transcriptional activity (Park et al., 2010). IRE1α signaling is attenuated after prolonged ER stress and this process is characterized by IRE1α cluster dissolution, IRE1α dephosphorylation and a decline in endoribonuclease activity (Li et al., 2010; Rubio et al., 2011). Additionally, the E3 ubiquitin ligase synoviolin increases IRE1α ubiquitination and degradation in synovial fibroblasts (Gao et al., 2008).

3.2.2. PERK Pathway

The second ER stress transducer is PERK, first described in mammalian pancreatic islet cells by Shi et al. This protein has a PEK-like catalytic domain, which phosphorylates the α subunit of the eukaryotic translation initiation factor-2 (eIF2α) (Shi et al., 1998). PERK possesses a luminal domain similar to that of IRE1, and a cytoplasmic portion that manifests protein serine/threonine kinase activity. As such, PERK is a member of the eIF2α kinase subfamily together with PKR, GCN2 and HRI (Harding et al., 1999). PERK is conserved in all known metazoans, but is absent in the *S. cerevisiae* genome. In *Drosophila melanogaster*, its homolog is DPERK (Pomar et al., 2003).

As mentioned, PERK is a type I transmembrane protein with a typical protein kinase structure that includes a large C-terminal lobe and a smaller N-terminal lobe linked by a short hinge loop. The C-terminal lobe is formed by a large activation loop, seven α-helices and two short β-strands. The N-terminal lobe contains three α-helices and five β-strands. A phosphate moiety was found in the electron density map at the position Thr980, and phosphorylation at this site is thought to stabilize the activation loop and the eIF2α-binding site in the αG helix (Cui et al., 2011).

When the UPR has not been activated, BiP/GRP78 is bound to the PERK luminal domain. Upon unfolded protein accumulation, BiP/GRP78 dissociates from PERK, and the loss of this interaction correlates with the formation of high molecular mass complexes of activated PERK. BiP/GRP78 overexpression attenuates this activation (Bertolotti et al., 2000).

ER stress leads to an increase in PERK's protein kinase activity and eIF2α phosphorylation, which competitively binds to eIF2β, a guanine nucleotide-exchange factor. This results in eIF2α-GDP to eIF2α-GTP exchange inhibition, the latter being a key component in the formation of the active 43S translation-initiation complex (Dever, 2002). Therefore, the PERK pathway inhibits general mRNA translation, decreasing global protein synthesis and reducing the ER load. PERK also contributes to UPR transcriptional activation and in ER-stressed PERK knockout cells, a characteristic decrease in mRNA responsible for normal UPR is observed (Harding et al., 2003).

Gene expression in response to eIF2α phosphorylation is conserved among eukaryotes. The transcription factor ATF4 is translationally induced because it has an upstream open reading frame (ORF) in its 5′-untranslated region. This upstream ORF, which under normal conditions, prevents translation of the true ATF4, is bypassed only when eIF2α is phosphorylated, and therefore, ATF4 translation occurs (Harding et al., 2000). One favored gene during this process is gadd153, also known as *chop*, an ER stress-induced proapoptotic factor (Fawcett et al., 1999). Also, the Nrf2 transcription factor is a substrate of PERK. In unstressed cells, Nrf2 is maintained in the cytoplasm by its association with Keap1. PERK-mediated phosphorylation triggers dissociation of Nrf2/Keap1 complexes and inhibits their reassociation, consequently causing Nrf2 nuclear import (Cullinan et al., 2003).

The entire range of PERK-dependent gene expression relies on eIF2α phosphorylation in Ser51, which is blocked in the Ser51Ala eIF2α mutant (Lu et al., 2004). In addition to its role in the ER stress, PERK plays an important role in activation of autophagy as a survival mechanism during episodes of nutrient deprivation, hypoxia and radiation (Ogata et al., 2006; Rouschop et al., 2010; Rzymski et al., 2010). These two functions allow PERK to regulate growth and survival (Bi et al., 2005; Blais et al., 2006).

After restoring homeostasis, activated PERK is dephosphorylated (Bertolotti et al., 2000) by mechanisms that remain to be determined. Also, active eIF2α is dephosphorylated by two phosphatases that function independently, namely CReP, a constitutively expressed phosphatase (Jousse et al., 2003), and GADD34, whose expression is induced by phosphorylated eIF2α (Novoa et al., 2001). A HSP40 family member, P58(IPK), also regulates PERK by binding to the kinase domain of the sensor and decreasing eIF2α phosphorylation. This regulation affects the expression of its downstream targets, decreasing the translation of the UPR target proteins BiP and CHOP. Moreover, P58(IPK) has also been implicated in the inhibition of PERK autophosphorylation (Yan et al., 2002).

3.2.3. ATF6 Pathway and Novel-Related Sensors

There are several cAMP response element (CRE)/ATF transcriptional factors inserted in the ER membrane regulating multiples genes associated with the UPR. The best known of these proteins is ATF6, which, together with PERK and IRE1, is the third major branch of the UPR.

The ATF6 transcription factor was until fairly recently considered a member of the protein family associated with the regulation of genes with CRE sequences (Hai et al., 1989). This 90 kDa protein has a bZIP domain (Hai et al., 1989; Zhu et al., 1997) for DNA binding after homo- or heterodimerization (Parker et al., 2001). Almost 10 years after initial description, the transcriptional activity of ATF6 was linked for the first time to mammalian UPR, because it was shown to bind to ER stress response elements (ERSE) in GRP promoter regions (Yoshida et al., 1998). ATF6 is now known as a single-pass type 2 transmembrane ER protein that transmits stress signals directly from the ER to the nucleus and thereby induces compensatory responses (Haze et al., 1999, 2001).

ATF6 has three structural domains, a luminal C-terminal, a transmembrane and a cytoplasmic N-terminal domain. Two isoforms of ATF6 have been described, ATFα (90 kDa) and ATF6β/G13/CREB-RP (110 kDa). In the luminal domain, ATF6 has Golgi localization sequences (GLS), two in the case of the ATF6α isoform (GLS1 and GLS2) and one (GLS2) in the case of the ATF6β isoform (Shen et al., 2002). Under basal conditions, ATF6 is retained in the ER via interaction with the chaperone BiP/GRP78 and calreticulin (Breckenridge et al., 2003; Shen et al., 2005). During ER stress conditions, ATF6 is transported on vesicles toward the Golgi apparatus (Schindler and Schekman, 2009), where it is sequentially cleaved by the Site-1 and then Site-2 Proteases (S1P and S2P, respectively) (Shen and Prywes, 2004). These intramembrane proteases were initially implicated in cleavage of the transcription factor steroid regulatory element-binding protein (SREBP), involved in lipid metabolism (Ye et al., 2000).

The N-terminal fragment of ATF6, p50ATF6, then translocates to the nucleus where it recognizes its consensus sequences and promotes transcription of UPR genes, such as the chaperones BiP/GRP78 and GRP94 (Yamamoto et al., 2004; Schröder and Kaufman, 2005; Yamamoto et al., 2007), the transcription factors CHOP (Ma et al., 2002) and XBP1 (Yoshida et al., 2001), as well as other proteins such as p58IPK/DNAJC3 (van Huizen et al., 2003), Herp (Kokame et al., 2001) and SERCA (Thuerauf et al., 2001). After the cleavage of ATF6α/β by S1P and S2P, two fragments are

generated from each isoform that translocate to the nucleus (p50ATF6α and p60ATF6β, respectively) (Thuerauf et al., 2004). ATF6 also plays a role in regulating ER volume increases in an XBP1-independent manner (Bommiasamy et al., 2009) and potentiates cellular adaptation to chronic ER stress (Wu et al., 2007). However, in ATF6α knockout mice, decreases in the basal expression of chaperones were not detected during either embryonic or postnatal development (Wu et al., 2007).

Interestingly, both ATF6 isoforms appear to play opposite roles in the UPR since ATF6β is a transcriptional repressor of the ATF6α signal and, therefore, a negative regulator of this branch of the UPR (Thuerauf et al., 2004). In contrast to ATF6β, ATF6α has a transactivation domain (TAD) in its N-terminal region with high homology to the section VN8 of the viral transcriptional factor VP16. This region was linked to an increase in the transcriptional activity of ATF6α and its degradation via the ubiquitin–proteasome system (Thuerauf et al., 2002). In vitro DNA interaction assays demonstrated that ATF6β binds to the consensus sequence in the BiP/GRP78 promoter region and blocks ATF6α binding (Thuerauf et al., 2007). Previous results have shown that ATF6β knockdown cells are more sensitive to tunicamycin-induced ER stress (Thuerauf et al., 2004). During the UPR, ATF6β levels regulate the intensity and the duration of responses to ATF6α, as well as susceptibility to cell death (Thuerauf et al., 2007).

Recently, it was determined that the ATF6β repressor effect depends on glycosylation. Nonglycosylated ATF6β is not cleaved and is retained in the ER membrane and, therefore, cannot function as a repressor (Guan et al., 2009). ATF6α is considered a very potent, but only transiently active transcription factor, as the increase in its transcriptional activity in response to unfolded proteins increases its own degradation by the proteasome (Thuerauf et al., 2002).

The subfamily of CREB3 transcription factors associate with ATF6 and function as sensors. These sensors are differentially expressed in different cell types and vary in their ability to initiate stress signals. At least five bZIP transcription factors related to ATF6 have been described. These are CREB3/Luman, CREB3L1/OASIS, CREB3L2/BBF2H7, CREB3L3/CREBH and CREB3L4/CREB4/AIbZIP/Tisp40. For more detailed information, interested readers are referred to two excellent recently published reviews (Asada et al., 2011; Chan et al., 2011).

Briefly, CREB3/Luman is expressed in some cell types, such as monocytes and dendritic cells. The mechanism and physiological conditions that lead to activation of this sensor are not entirely clear, but it is known to participate in the expression of various genes involved in the ERAD, like

Herp (Liang et al., 2006) and EDEM (DenBoer et al., 2005). Luman has been implicated in dendritic cell maturation, a process that is regulated by association with a complex containing the proteins DC-STAMP and OS9 (Eleveld-Trancikova et al., 2010). The transcriptional activity of Luman is regulated by differential interaction with the cofactor HCF-1 (herpes simplex virus-related host cell factor 1) or LRP (Luman recruitment factor), that favor or repress transcriptional activity, respectively (Audas et al., 2008).

On the other hand, OASIS/CREB3L1 is a transcription factor highly expressed in astrocytes and osteoblasts, and like the rest of subfamily members, is located in the ER membrane (Kondo et al., 2005). OASIS displays low homology with ATF6 in its luminal region but was described as a target for S1P/S2P proteases in response to ER stress (Murakami et al., 2006). OASIS-deficient mice suffer from severe osteopenia and spontaneous fractures generated by decreased ColIaI expression (Murakami et al., 2009). CREB3L2/BBF2H7 is a transcriptional factor analogous to OASIS that is highly expressed during the extension phase of long bones in the chondrocytes of the proliferating zone of cartilage. In addition, CREB3L2/BBF2H7 is expressed in other tissues, such as the lung and nervous tissue (Kondo et al., 2007). Sec23a, a protein required for the recruitment of Sec13/31 and other components of the COPII vesicles, important for vesicle trafficking from ER to Golgi, is controlled by CREB3L2 (Saito et al., 2009). CREB3L3/CREBH is expressed in hepatocytes and is also cleaved by S1P/S2P proteases. This transcription factor is known to regulate the expression of the gluconeogenic enzyme PEPCK in response to cyclic AMP and protein kinase A (Asada et al., 2011). Finally, CREB3L4/CREB4/AIbZIP/Tisp40 has been associated, among other things, with male germ cell development and the ER stress response during spermiogenesis (Adham et al., 2005).

3.3. Integral Response or Cross Talk between Different UPR Branches

ER stress sensors use different mechanisms and effectors to activate the UPR, but at some points, the three pathways communicate. One example is the close relationship between the IRE1α and ATF6 pathways. XBP1u not only functions as a negative regulator of XBP1 but also targets the active form of ATF6 to the proteasome (Yoshida et al., 2009), while ATF6, on the other hand, also controls the transcription of XBP1 (Yoshida et al., 2001). Additionally, ATF6 heterodimerizes with XBP1 to promote degradation of ERAD components (Yamamoto et al., 2007).

The PERK pathway is also linked to IRE1α and ATF6. Recently, a dominant negative form of PERK was shown to activate ATF6 and XBP1 (Yamaguchi et al., 2008). However, in another study, PERK was shown to facilitate both the synthesis and trafficking of ATF6 from the ER to the Golgi (Teske et al., 2011). All these studies underscore the notion that individual branches of the UPR are connected in ways that permit generating integrated responses to stress. Also, such connectivity explains the diverse defects associated with loss of any one of the sensors.

The integrated responses elicited by the three ER stress sensors contribute to either adaptation or death, but it remains unknown how each branch contributes to the final biological effect. A number of studies have attempted to resolve this issue. For instance, IRE1 and ATF6 activities are attenuated by persistent ER stress, while PERK signaling is not (Lin et al., 2007). Indeed, sustained PERK signaling impairs cell proliferation and promotes apoptosis. By contrast, signaling via IRE1α for an equivalent period of time enhances cell proliferation without promoting cell death (Lin et al., 2009), suggesting that the differential activation of PERK and IRE1α may determine cell fate following ER stress.

4. ENDOPLASMIC RETICULUM AND PROTEIN DEGRADATION

4.1. Endoplasmic Reticulum Quality Control

One of the main functions of the ER is the synthesis of proteins targeted to the secretory pathway. Proteins synthesized in the ER develop the appropriately folded native conformation following posttranslational modifications such as N-glycosylation and the formation of disulfide bonds. In order to export only correctly folded proteins, the ER is home to a variety of chaperones which facilitate protein folding. At the same time, they function as part of a quality-control system, which ensures that incompletely folded proteins are retained in the ER or targeted to degradation if appropriate folding cannot be achieved (Fig. 5.3).

The lectins calnexin and calreticulin play essential roles as chaperones in the ER quality-control system. As previously mentioned, most nascent polypeptide chains in the ER are N-glycosylated to facilitate the folding process (Schröder, 2008). Upon N-glycosylation, a branched oligosaccharide is attached to an asparagine residue on the target protein, thereby increasing the hydrophilicity of the nascent protein. Calnexin and calreticulin bind to glycosylated substrates containing a terminal glucose residue

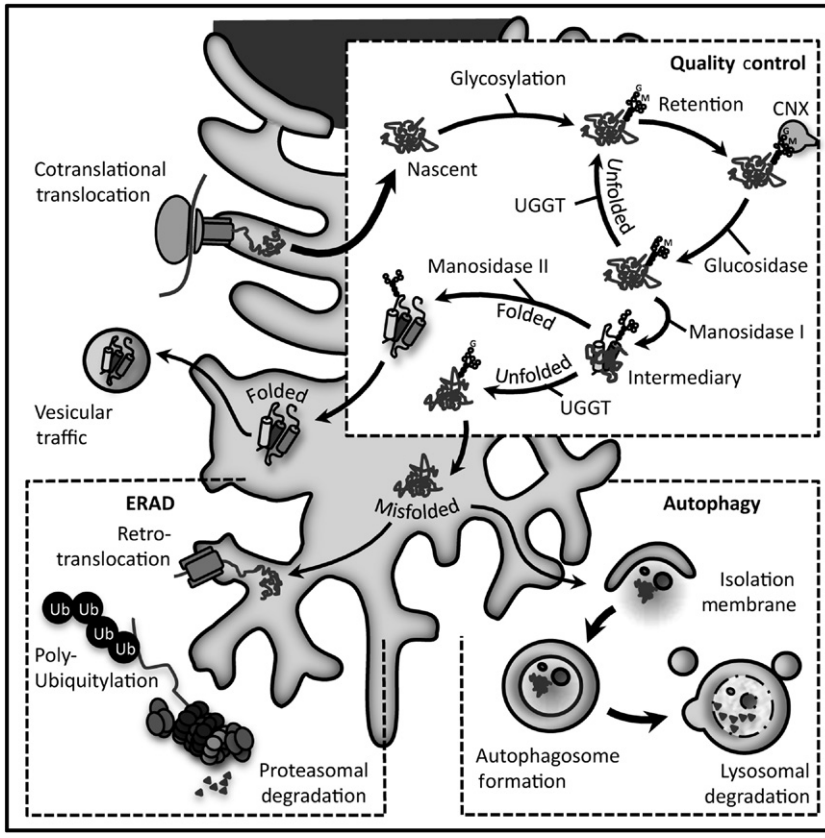

Figure 5.3 ***Proteins synthesized in the ER are subject to a quality control system***. The lectins, calnexin and calreticulin retain glycosylated proteins in the ER through re/deglucosylation cycles until they reach their native conformation. Folded proteins are exported through vesicle trafficking, while terminally misfolded proteins are degraded through retrotranslocation and the ubiquitin–proteasome system or autophagy.

and aid in protein folding by inhibiting protein–protein aggregation. In addition, calnexin and calreticulin trap partially folded or unfolded proteins in the ER (Hebert et al., 2005). While correctly folded proteins are deglucosylated by glucosidase II, then released from calnexin and calreticulin and exported to the Golgi apparatus, misfolded proteins are reglucosylated by the glycoprotein glucosyltransferase and retained in the ER (Hebert and Molinari, 2007). Folding attempts continue with each cycle of re/deglucosylation up until demannosylation occurs via α-mannosidase I. Once demannosylated, glycoproteins become a weaker substrate for glucosidase II and glucosyltransferase, thus preventing them from entering new

cycles of glucosylation (Hebert et al., 2005). Other lectins like EDEM work as acceptors for terminally misfolded proteins accelerating the demannosylation process (Olivari et al., 2006). Overexpression of EDEM greatly accelerates protein degradation while its downregulation prolongs folding attempts and delays protein degradation (Molinari et al., 2003). Altogether, the ER quality-control system ensures that correctly folded proteins leave the ER while incorrectly or terminally misfolded proteins are retained in the ER and degraded.

4.2. Misfolded Protein Degradation through ERAD and Autophagy

Terminally misfolded proteins can be degraded through two different pathways: The ubiquitin–proteasome pathway named ERAD and the lysosomal pathway termed *macroautophagy* (hereafter referred to as autophagy). The ERAD pathway consists in the retrotranslocation, polyubiquitination and proteasomal degradation of misfolded proteins from the ER (Meusser et al., 2005). Terminally misfolded proteins are translocated across the ER membrane into the cytoplasm where they are covalently bound to ubiquitin through a lysine residue. Attachment of multiple ubiquitins yields polyubiquitin chains, which are a signal for degradation inside a cylinder-shaped protein complex, termed the *proteasome*. Once inside the proteasome, the protein becomes a substrate for the multiple proteolytic activities present, which degrade the target protein and recycle the polyubiquitin side chain (Vembar and Brodsky, 2008). On the other hand, during autophagy, different molecular components such as proteins, lipids or even entire organelles are sequestered into double-membrane vesicles, which then fuse with lysosomes, to allow the degradation of vesicle contents (He and Klionsky, 2009).

Although ERAD has been described as the primary source for ER misfolded protein degradation, recent reports have shown that autophagy is involved in the removal of certain ER proteins such as α1-antitrypsin. These observations identified autophagy as an alternative cellular strategy to eliminate unfolded proteins (Yorimitsu et al., 2006; Yorimitsu and Klionsky, 2007).

4.2.1. ERAD

The exact mechanism by which ERAD substrates are retrotranslocated across the ER membrane is still a matter of debate. Several proteins have been identified that play an essential role in this process by creating a channel

in the ER membrane that permits retrograde transport of misfolded proteins back to the cytoplasm (Meusser et al., 2005). The Sec61 complex has been proposed as a possible channel for protein retrotranslocation (Wiertz et al., 1996) although this complex was first described as a channel through which nascent polypeptides are imported into the ER (Mothes et al., 1994; Matlack et al., 1998). Another possible candidate retrotranslocation channel is Derlin-1, which forms a complex with the ubiquitin ligase Hrd-1 along with other adaptor proteins (Carvalho et al., 2010). Derlin-1 has the ability to form structures that span the ER membrane, suggesting that this protein may also function as a channel for ERAD retrotranslocation (Crawshaw et al., 2007).

Research in *S. cerevisiae* showed that protein substrates are recognized and degraded differently, depending on whether their misfolded domain is cytosolic (ERAD-C), transmembrane (ERAD-M) or luminal (ERAD-L) (Carvalho et al., 2006). While ERAD-C substrates are rapidly degraded through the ubiquitin ligase Doa10p (Swanson et al., 2001), ERAD-L and ERAD-M depend on the Hrd ubiquitin ligase complex for degradation (Bordallo et al., 1998; Schäfer and Wolf, 2009). Studies in yeast using different glycosylated and nonglycosylated degradation substrates identified the basic components of the Hrd ubiquitin ligase complex and its adapter protein functions. The basic structure of this complex is formed by the ubiquitin ligase Hrd1p, the substrate adapter protein Hrd3p, the luminal substrate adapter proteins Der1p and Usa1p, the ubiquitin conjugating enzyme Ubc7 and the glycan substrate adapter Yos9p (Carvalho et al., 2006; Mehnert et al., 2010). Different mammalian homologs of Hrd complex proteins have been identified by correlating with functions in yeast (Mehnert et al., 2010). Among these mammalian homologs, Herp, an ubiquitin-like ER membrane protein, has been recently found to regulate Hrd1-dependent ubiquitination (Kokame et al., 2000; Kny et al., 2011). Recent studies have shown that Herp is involved in regulating the degradation of multiple ER substrates such as immature nicastrin and α-1 antitrypsin (Kny et al., 2011; Marutani et al., 2011).

Retrotranslocation across the ER membrane of misfolded, polyubiquitinated proteins is an active process that requires ATP hydrolysis. The AAA-ATPase p97 and its adapter proteins Ufd and Npl4 are necessary to generate the driving force required for misfolded protein extraction (Ye et al., 2001). In mammalian cells, p97 binds Derlin-1 via VIMP and other cofactors, thereby permitting recruitment of misfolded proteins to the ER membrane, their export and proteasomal degradation in the cytoplasm (Ye et al., 2004).

4.2.2. Autophagy

Autophagy is induced by nutrient deprivation. The kinase AMPK responds to low energy levels of the cell, by sensing the declining ATP/AMP ratios. Once activated, AMPK phosphorylates mTOR, a master regulator of protein synthesis. mTOR normally functions as a Ser/Thr kinase, which hyperphosphorylates Atg13, thereby impeding the activation of autophagy. mTOR phosphorylation by AMPK reduces Atg13 phosphorylation, thus permitting its interaction with the kinase Atg1 and activation of a phosphorylation cascade that leads to the recruitment and activation of a series of Atg proteins indispensable for autophagy induction (He and Klionsky, 2009; Jung et al., 2010). Among the most important Atgs are LC3/Atg8 and Beclin-1/Atg6. Beclin-1 is part of a protein complex termed PI3K-III, which is important for the nucleation, recruitment and expansion of the double membrane that eventually sequesters the substrates destined for degradation. In normal conditions, Beclin-1 interacts with its inhibitor Bcl-2. Beclin-1 dissociation from Bcl-2 is indispensable for PI3K-III function and autophagy activation (Kang et al., 2011). During the autophagic pathway, LC3 is processed and attached to the autophagosome membrane by conjugation with phosphatidylethanolamine (Klionsky et al., 2007).

As indicated, recent work has shown that autophagy is induced under ER stress conditions to protect cells against death (Ogata et al., 2006; Pankiv et al., 2007). Work in yeast, utilizing classic ER stress inducers, such as DTT or tunicamycin, revealed that autophagy was induced, as assessed by LC3 processing and Atg1 kinase activity (Yorimitsu et al., 2006; Yorimitsu and Klionsky, 2007). This correlates with other work in which Hac1, an XBP1 yeast ortholog, induced important autophagy genes such as Atg5, Atg7 and Atg8 under ER stress conditions (Bernales et al., 2006). Other studies in mammalian cells showed that autophagy is a key component in the degradation of α1-antitrypsin retained inside the ER (Teckman and Perlmutter, 2000). ER-mediated regulation of autophagy has been linked to the PERK and IRE1 pathways. PERK phosphorylation of eIF2α is involved in Atg12 upregulation and LC3 conversion during polyglutamine aggregate-induced autophagy (Kouroku et al., 2007). PERK phosphorylation of eIF2α is also involved in the degradation of mutant dysferlin aggregates via LC3 conversion and autophagy (Fujita et al., 2007). Other studies have implicated the IRE1 pathway in ER stress-induced autophagy. IRE1 recruits the protein factor TRAF2, which activates JNK that phosphorylates Beclin-1, thereby allowing dissociation from its repressor Bcl-2 (Ogata et al., 2006). ER calcium release is also thought to participate in autophagy induction

(Høyer-Hansen et al., 2007). CaMKKβ is activated by ER stress-induced calcium release, and in turn activates AMPK, leading to the inhibition of mTOR and induction of autophagy (Høyer-Hansen et al., 2007; Høyer-Hansen and Jäättelä, 2007).

4.2.3. Examples of ER Proteins Degraded by ERAD and/or Autophagy

Supporting the notion that autophagy is as important as ERAD for degradation of terminally misfolded ER proteins, recent studies have shown that dual degradation mechanisms exist for certain proteins, such as α1-antitrypsin and mutant fibrinogen. Both mutant proteins form aggregates in the ER, which, if not cleared, promote cell death. Recent studies have shown that high levels of α1-antitrypsin or mutant fibrinogen can saturate the ERAD pathway, leading to the accumulation of protein aggregates that must be cleared through the autophagy pathway (Kruse et al., 2006a, 2006b). Another example where dual degradation pathways are observed is dysferlin. While wild-type dysferlin is degraded through the ERAD pathway, mutant dysferlin forms protein aggregates that impair ERAD and are degraded through autophagy (Fujita et al., 2007). ERAD and autophagy also play a role in serpin and procollagen degradation. ERAD seems to have a selective role in mutant neuroserpin degradation, while autophagy degrades all forms of neuroserpins. Thus, autophagy becomes an important clinical target since induction of this degradation pathway could help to overcome the accumulation of mutant protein aggregates (Kroeger et al., 2009). Autophagy degrades misfolded procollagen that accumulates as trimers, while the misfolded procollagen monomers are degraded through the ERAD pathway (Ishida and Nagata, 2009; Ishida et al., 2009). Interestingly, EDEM1, a protein involved in quality control and ERAD, is also degraded through autophagy, suggesting that the ERAD pathway may be regulated by autophagy (Le Fourn et al., 2009).

4.3. ER-phagy

ER-phagy, as well as mitophagy and peroxiphagy, are organelle-specific autophagic processes that degrade ER, mitochondria or peroxisomes, respectively. ER-phagy is a specific process, which uses several autophagy genes induced by ER stress. Normally, ER stress increases the volume of the ER in order to inhibit protein–protein aggregation. ER-phagy is the sequestration of ER into double-membrane vesicles. Interestingly, both the sequestered content and the vesicle-forming membranes are of ER origin, which suggests that the ER engulfs itself in order for ER-phagy to

proceed. Even though autophagy is a degradation process in which the autophagosome fuses with the lysosome to permit degradation of the autophagosome content, ER-phagy is not a degradation process since mutants lacking vacuolar proteases are able to sequester the ER. Thus, the function of ER-phagy seems to involve sequestering damaged portions of the ER, misfolded protein aggregates that cannot be degraded by other means, as well as reducing the volume of the ER once ER stress is over (Bernales et al., 2006, 2007).

In summary, secretory proteins and most integral membrane proteins enter into ER for proper folding and covalent modifications to assemble into complexes of higher order. ER-resident chaperones and other modifying enzymes assist proteins to achieve their active and final, three-dimensional conformation. Only properly folded and assembled proteins are allowed to exit the ER, thereby providing an exquisite quality-control mechanism that maintains the fidelity of protein synthesis. The process is regulated at multiple levels ensuring that ER folding capacity is not overwhelmed, thus maintaining ER homeostasis. At the center of this regulation is the phylogenetically conserved process of the UPR, a key signaling pathway that triggers comprehensive remodeling of the ER and the biosynthetic pathway according to the different cellular needs. In parallel, autophagy is another important degradation pathway normally induced by nutrient deprivation and more recently, under ER stress conditions, to protect cells from death. Two reports (Bernales et al., 2006, 2007) that describe the new process of ER-phagy provide direct evidence that the ER can serve as a membrane source for autophagosome formation. Further, they indicate that this process entails engulfment of the ER by itself, targeting the ER as the major organelle involved in the regulation of cellular component degradation. As such, it plays an important role in protein- and organelle-degradation functionality of the ER critical to homeostatic cellular control.

5. UPR AND CELL METABOLISM

The main goal of the UPR is to restore the equilibrium between protein load and folding capacity of the ER. From a metabolic point of view, protein folding in the ER is a demanding process, due to its high-energy requirements (Fig. 5.4). For example, saccharides are needed for the N-glycosylation of client proteins, reductive equivalents are consumed in the formation of disulphide bonds and an appropriate supply of ATP is essential for calcium accumulation in the ER and chaperone activity.

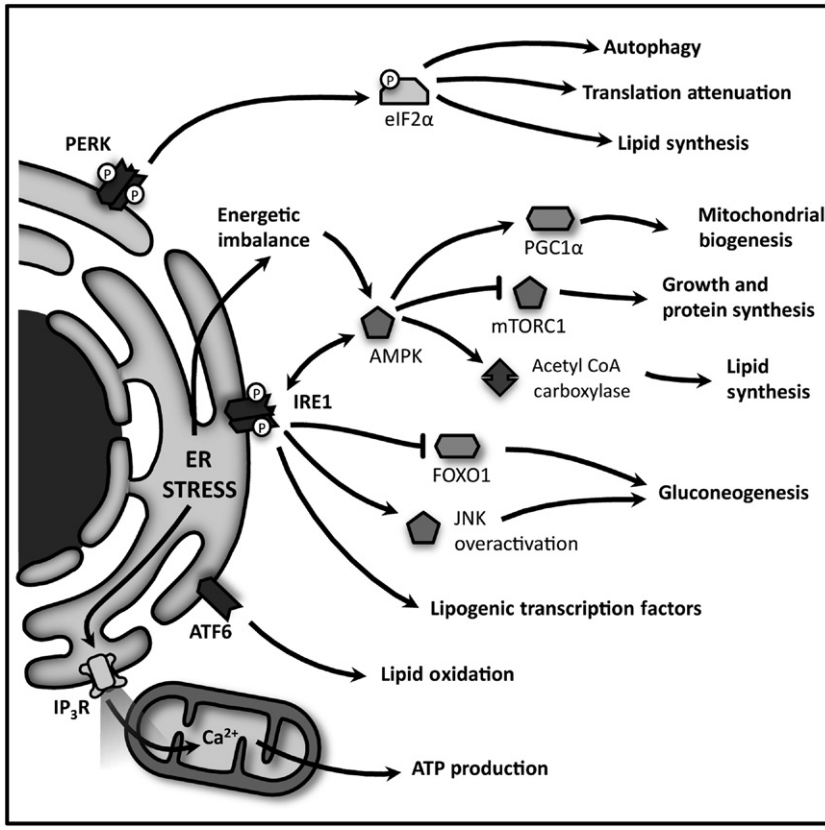

Figure 5.4 ***The ER modulates important metabolic pathways during ER stress***. PERK induces global attenuation in protein anabolism, and also favors lipid synthesis, both effects via eIF2α phosphorylation. IRE1 cooperates with these responses and stimulates mitochondrial biogenesis through the regulation of master metabolic switches, such as PGC1α, mTOR, AMPK and FOXO1. ATF6, on the other hand, stimulates lipid utilization. Calcium released by IP3R increases mitochondrial activity during ER stress, in order to revert energy imbalance.

5.1. UPR-Mediated Regulation of Master Metabolic Switches

The best-known effect of UPR on cellular metabolism is the attenuation of general protein translation via eIF2α phosphorylation. Such negative regulation not only reduces protein load in the ER but also increases ATP availability for processes such as protein folding and degradation.

As already mentioned, conditions of low nutrient supply are known to induce the UPR as well as autophagy. The relationship between these two responses becomes clear considering that nutrient deprivation increases

protein misfolding, which is a potent trigger of both processes. However, cross talk between the UPR and master metabolic regulators has received little attention until recently. AMPK has been shown to require the endoribonuclease activity of IRE1 for nitric oxide-induced signaling (Meares et al., 2011). Moreover, stimulation of the IRE1 endoribonuclease activity with the flavonoid quercetin also engages IRE1-dependent AMPK activation. This signaling pathway is important for maintaining cellular metabolism because it regulates the activity of critical proteins, such as acetyl-CoA carboxylase, PGC-1α and mTORC1. This cooperative effect is of particular interest in tissues with high metabolic activity, such as the heart. The AMPK activators AICAR or metformin relieve hypoxia/reoxygenation injury, a process known to trigger apoptosis in cardiomyocytes, by generating ER stress (Yeh et al., 2010). This response is achieved by the concomitant decrease in proapoptotic branches of the UPR (CHOP and caspase-12) while retaining an adaptive increase in BiP/GRP78 protein levels. Taken together, this evidence suggests that AMPK signaling may cooperate with, or even be part of the adaptive steps of UPR, by diminishing death-inducing irreparable damage. However, many details of this complex cross talk process still need to be unraveled.

When ER stress is induced by increased protein load, instead of decreased nutrients, the role of mTOR has been shown to be important. Such is the case for plasma cells, which secrete large amounts of immunoglobulins when they are stimulated with LPS. These cells, upon induction of ER stress, attenuate the mTOR pathway, leading to reduced protein synthesis. This reduction is important for cellular adaptation as B cells with elevated mTOR activity are prone to undergo apoptosis upon LPS stimulation (Goldfinger et al., 2011). A second example that shows the preponderance of mTOR during ER stress has been established in a rat model of minimal-change disease, which is characterized by podocyte ER stress-dependent proteinuria. In this model, mTORC1 activation was shown to precede UPR, leading to a decrease in the ATP/ADP ratio and activation of AMPK, presumably due to an increase in energy-consuming processes, such as cell growth and proliferation. Pretreatment with the mTORC1 inhibitor, everolimus, prevented the reduction in ATP levels, AMPK activation and UPR, thus inhibiting proteinuria (Ito et al., 2011). Studies in tumor cells have shown that constitutive activation of mTOR induces ER stress, which is part of a negative feedback loop emerging from growth factor receptors upstream of mTOR (Ozcan et al., 2008). On the other hand, during ER stress, mTORC1 triggers apoptosis by suppressing Akt

activity, and selectively inducing the IRE1-JNK pathway (Kato et al., 2011). These observations highlight the delicate balance that exists between cellular energy sensing and ER stress.

5.2. UPR Regulation of Lipid Metabolism

ER stress is known to stimulate lipogenesis through the UPR, thus providing lipids for ER expansion, a hallmark of cellular adaptation. ER stress activates, for example, SREBP1 (Wang et al., 2005), as well as SREBP2 (Colgan et al., 2007), two master transcriptional regulators of fatty acid and cholesterol biosynthesis. Relief of ER stress by overexpression of BiP/GRP78 inhibits SREBP1 activation in the liver of ob/ob mice, highlighting the importance of the UPR in the control of lipid metabolism (Kammoun et al., 2009).

As mentioned in previous sections, the IRE1/XBP1 pathway was first shown to be important for the differentiation of highly secretory cells, such as antibody-secreting B cells. Likewise, XBP1-deficient cells are reportedly deficient in adipogenic differentiation (Sha et al., 2009). XBP1 was shown to be directly downstream the essential adipogenic factor C/EBPβ, and splicing of this factor is indispensible for the development of the adipose phenotype. One mechanism by which XBP1 promotes lipogenesis is through activation of enzymes of the CDP-choline pathway, thus leading to increased phosphatidylcholine biosynthesis and ER biogenesis (Sriburi et al., 2004, 2007). In the liver, the IRE1/XBP1 pathway directly controls genes involved in fatty acid synthesis in response to carbohydrate diet, while hepatic deletion of XBP1 causes hypocholesterolemia and hypotriglyceridemia (Lee et al., 2008). Furthermore, hepatocyte-specific IRE1 deletion predisposes mice to ER stress-induced hepatosteatosis, suggesting that this UPR sensor is also important for intracellular lipid secretion and accumulation (Zhang et al., 2011).

PERK has also been demonstrated to be important for lipogenic tissue development since knockout mice have impaired mammary gland lipogenesis during pregnancy, which results in reduced free fatty acid content of the milk (Bobrovnikova-Marjon et al., 2008). Loss of PERK also resulted in a decrease in key lipogenic enzymes, such as stearyl-CoA desaturase-1, fatty acid synthase and ATP citrate lyase, due to excessive activation of SREBP1, the master regulator of lipid homeostasis within the cell. The PERK pathway is also important for development of hepatosteatosis in mice fed a high-fat diet. Reduction of eIF2α phosphorylation results in a lower expression of PPARγ and C/EBP, key transcriptional regulators of lipid synthesis (Oyadomari et al., 2008).

On the other hand, ATF6 has also been implicated in lipid metabolism. ATF6 knockout mice treated with tunicamycin develop hepatosteatosis to a higher degree than wild-type mice. In these animals, a more pronounced decrease in the expression of genes related to lipid handling, such as PGC1α, PPARα and SREBP1, is observed, which results in increased lipid accumulation in the liver. However, unlike the other UPR pathways, ATF6 seems to be more important for fatty acid oxidation than lipid biosynthesis (Rutkowski et al., 2008).

5.3. UPR Regulation of Carbohydrate Homeostasis

In rat liver, it was recently shown that physiological postprandial increases in glucose and lipids cause ER stress (Boden et al., 2011), suggesting that the UPR may participate in carbohydrate metabolism. FOXO1, a transcription factor that promotes hepatic gluconeogenesis and inhibits glucose utilization (Zhang et al., 2006), is regulated by XBP1 in an ER stress response-independent manner (Zhou et al., 2011). Spliced XBP1 interacts with FOXO1 to enhance proteasome-mediated degradation. This effect is observed even at low levels of XBP1 that are unable to induce UPR-related genes. This evidence shows that UPR sensors control carbohydrate metabolism and diminish glucose production. Alternatively, another line of evidence links ER stress-induced JNK hyperactivation to increased FOXO1 activity and increased hepatic gluconeogenesis (Lim et al., 2009). This mechanism may be important for regulation of blood sugar during long periods of hypoglycemia since this condition has been shown to induce ER stress in the liver, thereby linking the UPR and glucose production (Gonzales et al., 2008). These contradictory results suggest that the connection between UPR and hepatic glucose handling is complex and further studies are required to fully dissect the details.

The pathological implications of UPR for glucose metabolism in different organs have been extensively documented in recent years, due to its importance in understanding type 2 diabetes. This issue will be covered in the next section.

5.4. ER Stress-Modulated Mitochondrial Metabolism

While the role of the ER as a physiologically relevant calcium store is well established, a similar role for mitochondria has been long debated. Calcium ions are known to be taken up by mitochondria and to accumulate in the mitochondrial matrix (Deluca and Engstrom, 1961; Vasington and Murphy, 1962), but the relevance of this process remained unclear. Moreover,

evidence suggesting the existence of a low-affinity calcium uptake system questioned the physiological relevance of mitochondria in calcium handling.

For many years, calcium accumulation and protection of the cell against calcium overload was considered to be the only function of mitochondria in the control of intracellular calcium metabolism. However, this view was challenged by observations showing that the activity of pyruvate, isocitrate and α-ketoglutarate dehydrogenases in permeabilized or homogenized mitochondria was enhanced by calcium in a direct or an indirect manner (McCormack et al., 1990; Carafoli, 2010). Interestingly, agonist-induced calcium release can lead to improved mitochondrial function as evidenced by increased ATP production after restoring normal mitochondrial calcium levels (Jouaville et al., 1999). Additionally, also many other mitochondrial processes, such as fatty acid oxidation, amino acid catabolism, F1-ATPase manganese superoxide dismutase activity, aspartate and glutamate carrier, as well as the adenine-nucleotide translocase activity, are regulated by mitochondrial calcium (McCormack et al., 1990; Hayashi et al., 2009). Moreover, Cárdenas et al. (2010) recently showed that basal IP3R activity was required to control mitochondrial bioenergetics. Absence of this calcium transfer results in enhanced phosphorylation of pyruvate dehydrogenase and AMPK activation, which in turn activates autophagy as a prosurvival response. Thus, constitutive IP3R-mediated calcium release to mitochondria is required for efficient mitochondrial respiration and maintenance of normal cellular bioenergetics.

Work from our group performed in HeLa cells demonstrated that early stages of ER stress increase physical coupling and calcium transfer from ER to mitochondria, leading to augmented mitochondrial bioenergetics as a means of adaptive ATP production (Bravo et al., 2011b). Physical coupling between ER and mitochondria is observed upon treatment with different ER stressors (tunicamycin, brefeldin A and thapsigargin). However, it remains unknown to what extent the UPR is involved in these events.

Additionally, a recent study performed in skeletal muscle showed that adaptation of muscle fibers to acute exercise is mediated by the UPR. ATF6 is required for the recovery process, involving the coactivation of PGC1α (Wu et al., 2011). Such cross talk between the UPR and PGC1α is a potential mechanism that may explain how ER stress-mediated control of mitochondrial metabolism is achieved, given the importance of PGC1α in mitochondrial biogenesis and fatty acid oxidation (Koves et al., 2005; Safdar et al., 2011). Despite such insights, the field of ER-modulated mitochondrial

metabolism is still rather nascent, and further studies are required to determine their relevance to the development of human diseases. Some of these pathological implications will be discussed in the next and final sections.

6. UPR AND PATHOLOGIES

Pathophysiological alterations in cellular calcium along with ER stress signaling are hallmarks of a broad variety of pathologies such as type-2 diabetes mellitus (T2DM), insulin resistance, cardiovascular diseases and Alzheimer disease (AD) (Kelley et al., 2002; Lim et al., 2009). These

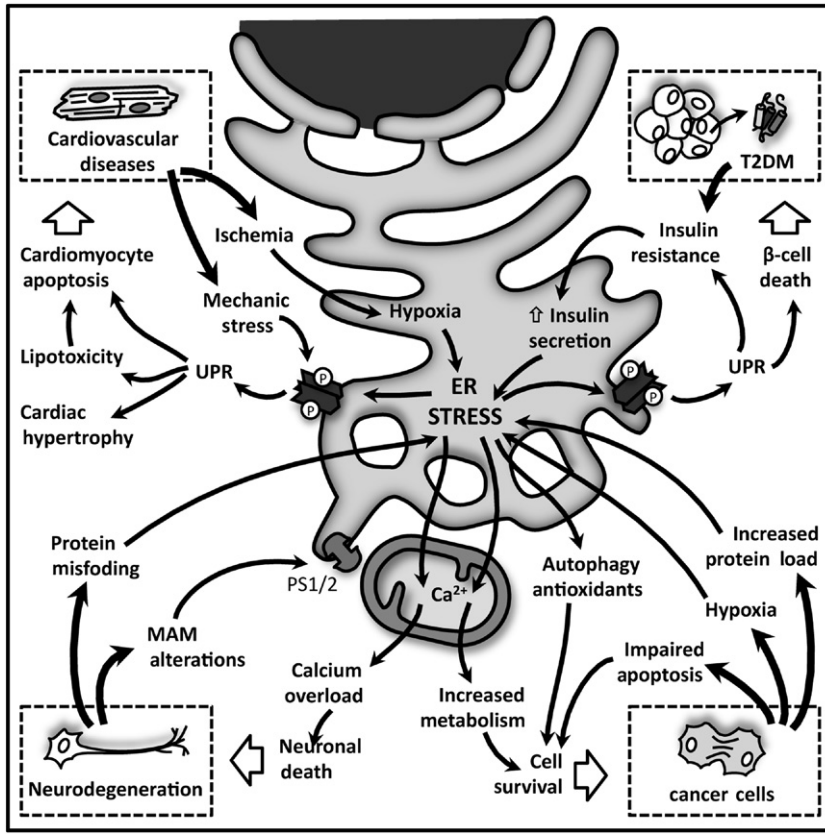

Figure 5.5 ***ER and pathologies***. ER stress and alterations in cellular calcium characterize diverse pathologies such as type-2 diabetes mellitus, insulin resistance, and some cardiovascular and neurodegenerative diseases. These pathologies are also characterized by marked decrease in cellular metabolism, which is accompanied with protein misfolding and aggregation, and some characteristic hallmarks of UPR. However, the molecular mechanisms linking all these processes are not yet fully understood.

pathologies are also characterized by a marked decrease in cellular metabolism; however, the molecular mechanisms responsible for this dysfunction are not fully understood. As discussed previously, alterations in the contacts between ER and mitochondria, and more importantly, in calcium transfer between these two organelles, could lead to mitochondrial dysfunction and the metabolic imbalance observed in these diseases (Fig. 5.5). Moreover, recent studies have also linked ER–mitochondria communication to cancer growth and progression. The influence of both organelle interplay and UPR signaling on human pathologies will be discussed subsequently.

6.1. UPR and Diabetes

Diabetes mellitus (DM) is considered a multifactorial pathology and is associated with obesity, dyslipidemia, endothelial dysfunction, inflammation and hypertension (Petersen and Shulman, 2006). T2DM is one of the most common diseases in the developed world and it is now considered as a global health burden (Zimmet et al., 2001). Peripheral insulin resistance, deregulated hepatic glucose production and inadequate insulin secretion characterize T2DM. Furthermore, this disease involves defects in insulin signaling due to reduced insulin receptor function and downstream phosphorylation events (Lee and White, 2004). Recent reports using genetically obese (ob/ob) or high-fat diet (HFD)-induced obese mice identified an increase in UPR markers in the liver and adipose tissues (Ozcan et al., 2004). Moreover, elevated ER stress markers, such as GRP78, XBP1s, phosphorylated eIF2a and JNK, are detected in the liver and adipose tissue of obese insulin-resistant individuals (Boden et al., 2008). Additionally, JNK activation via UPR is associated with insulin resistance via phosphorylation of IRS-1. Hotamisligil's group described a causal link between ER stress and the development of metabolic diseases like insulin resistance and T2DM. Using different cell culture and mouse models, they showed that obesity causes ER stress. Moreover, mice deficient in the XBP-1 protein develop insulin resistance, demonstrating that ER stress is a central feature of peripheral insulin resistance and T2DM at the molecular and cellular level (Ozcan et al., 2004). In another more recent study, XBP-1 was shown to interact with the transcription factor FOXO1, a key regulator of gluconeogenesis, and promote its proteasomal degradation. Moreover, hepatic overexpression of XBP-1 results in significantly reduced blood glucose levels and increased glucose tolerance in mouse models of insulin resistance and type 1 and 2 diabetes. All these changes are accompanied by a reduction in hepatic FoxO1, revealing that XBP-1 regulates glucose homeostasis in response to ER stress (Zhou et al., 2011).

Enhanced gluconeogenesis is another important component of insulin resistance in T2DM. Interestingly, the induction of acute ER stress results in reduced hepatic glucose output mediated by ATF6 interaction with the CREB-regulated transcription coactivator 2 (CRTC2) that releases CREB, which regulates fasting gluconeogenesis (Wang et al., 2009c). Moreover, in CRTC2-knockout animals, decreased fasting gluconeogenesis and improved insulin sensitivity are observed upon diet-induced obesity (Wang et al., 2010). Additionally, chronic ER stress also increases gluconeogenesis. Obese mice have lower levels of hepatic ATF6 and XBP-1, which reduces the interaction of ATF6 with CRTC2, thereby favoring upregulation of the gluconeogenesis program (Wang et al., 2009c). Furthermore, XBP-1 reduction contributes to enhanced JNK activation that triggers insulin resistance (Ozcan et al., 2004).

PERK, the third branch of the UPR, acutely and chronically phosphorylates eIEF2α during hepatic ER stress (Ozcan et al., 2004; Lin et al., 2007), increasing gluconeogenesis via the translational activation of C/EBP. Additionally, the dephosphorylation of hepatic eIEF2α improves insulin sensitivity in HFD mice (Oyadomari et al., 2008). eIF2α is also implicated in hepatic lipogenesis. Thus, ATF6 knockout and chronic dephosphorylation of eIF2α in a mouse model decrease C/EBP expression and downstream lipogenic transcription factor activity (Oyadomari et al., 2008; Rutkowski et al., 2008). Finally, ER stress contributes to hepatic fat accumulation through changes in the nuclear localization of SREBP1c by an insulin-independent pathway (Kammoun et al., 2009).

During the development of T2DM, insulin production by β-cells is increased to compensate for insulin resistance. This increase in insulin demand, together with increased fatty acid levels and hyperglycemia, activates UPR in pancreatic cells (Cnop et al., 2005). Different kinds of mutations are associated with diabetes. Missense mutations that interrupt disulphide bridge formation and correct proinsulin folding cause neonatal diabetes (Støy et al., 2007; Colombo et al., 2008). Moreover, mutations in several UPR genes, like eif2ak3 (which codes for PERK) and wsf1, are considered risk factors for the development of diabetes, in the Wolcott–Rallison and Wolfram syndromes, respectively (Inoue et al., 1998) (Delépine et al., 2000). Prolonged T2DM can activate the UPR by hyperglycemia. In contrast, free fatty acids (FFA) may trigger ER stress early in diabetes. Several groups have demonstrated that chronically high glucose levels cause ER stress (Wang et al., 2005; Lipson et al., 2006; Elouil et al., 2007). For example, cultured rat pancreatic islets exposed to high glucose (30 mM)

increase the mRNA levels of Grp78, Grp94, Edem and Chop (Elouil et al., 2007). However, a study in MIN6 insulinoma cells exposed to low or high glucose failed to induce the UPR response (Webb et al., 2000). Besides, high glucose levels are also associated with an increased level of ROS and a reduction in β-cell function (Robertson, 2004; Robertson et al., 2004). Although ROS can induce ER stress (Malhotra and Kaufman, 2007), they also cause numerous other cellular effects.

As for hyperglycemia, elevated levels of FFA are also linked to obesity and contribute to the development of T2DM (Robertson et al., 2004). Exposure of human islets to palmitate induces ER stress and an NF-kB-mediated inflammatory response (Igoillo-Esteve et al., 2010). Furthermore, the treatment of β-cells with palmitate causes ER distention and activation of the three branches of the UPR. This activation may contribute to FFA-induced β-cell death through CHOP upregulation and the activation of JNK as well as the caspase-12 cascade (Kharroubi et al., 2004), which rapidly degrades carboxypeptidase E, an enzyme involved in the insulin processing. FFA also induces the depletion of ER calcium and impedes protein trafficking by ceramide synthesis (Boslem et al., 2011). All these events contribute to ER stress by the accumulation of unprocessed proinsulin molecules in the secretory pathway (Jeffrey et al., 2008).

Finally, obesity and occidental diet are associated with elevated plasma levels of glucose and FFA. Both factors contribute to the progression of T2DM by promoting pancreatic β-cell death (Cunha et al., 2008, 2009). While CHOP has a proapoptotic effect in β-cells, PERK antagonizes the apoptosis antagonizing transcription factor (AATF), which regulates Akt1 (Ishigaki et al., 2010). Additionally, the upregulation of C/EBP sensitizes cells to apoptosis (Matsuda et al., 2010). Although our understanding of how chronic ER stress contributes to the development of diabetes is improving, further studies are needed to fully appreciate the existing connections between UPR and the metabolic nodes regulated by mTOR and AMPK, as well as how the drugs metformin and resveratrol affect ER stress.

6.2. UPR and Cardiovascular Diseases

6.2.1. Ischemia/Reperfusion

All three branches of the UPR are activated in ischemic heart disease (Glembotski, 2007). In an in vitro model using HL-1 cells, exposure to 8 h of hypoxia triggered phosphorylation of PERK and expression of CHOP (Perman et al., 2011). Also, in rat neonatal cardiomyocytes, ischemia stimulated significant upregulation of BiP/GRP78 and ATF6, as well as an

increase in XBP1 splicing (Thuerauf et al., 2006). The majority of these and other in vitro findings have been replicated in animal models and clinical studies under various conditions (Thuerauf et al., 2006; Severino et al., 2007; Toko et al., 2010; Perman et al., 2011). Studies in wild-type animals showed that ATF6 transcription was increased 6 h after ischemia (Perman et al., 2011). Alternatively, Thuerauf et al. (2006) found that BiP/GRP78 levels were elevated even 4 days after ischemic injury in cardiomyocytes.

Under ischemic conditions, the lack of oxygen and nutrients poses a serious threat to cardiomyocyte viability. At the mechanistic level, insufficient ATP impairs disulfide bond formation and fosters inappropriate calcium handling, both processes that have been suggested to trigger UPR in heart. Disulfide bond formation is critical for folding of secreted proteins, and the ER-resident chaperones involved, Ero1, PDI and ERp57, require oxygen as a final electron acceptor for the serial redox reactions required for this process (Anelli and Sitia, 2008). Correct glycosylation of membrane proteins is required for both trafficking and proper biological function. Recent studies suggest that insufficient ATP availability affects UDP-sugar formation and triggers ER stress by interfering with glycosylation. The sudden drop in nutrient flux during ischemic heart disease leads rapidly to energy and ATP shortage. Additionally, high calcium levels in the ER are required for chaperones such as BiP/GRP78 to aid in protein folding. The pathological changes in ER calcium levels during ischemia due to decreased SERCA2 activity can effectively stimulate ER stress (Liu et al., 2011).

Although activation of the UPR in ischemic heart disease is well established, the precise biological role of the UPR in this process remains unclear, since available evidence indicates that activation of the UPR can be either beneficial or detrimental. Most studies have attempted to address this question by evaluating activity of proapoptotic branches of the UPR following genetic or pharmacological manipulation. For instance, studies with VLDL-deficient rats established that protection of the heart from ischemic insult correlated with a decrease in caspase activity and apoptotic cell death (Perman et al., 2011). Likewise, this correlation was evident in a rat ischemia model where BiP/GRP78, CHOP and caspase-12 were evaluated (Song et al., 2011). These studies suggest that ER stress during ischemia stimulates cell death, and inhibition of ER stress may ameliorate cardiomyopathy. However, evidence for a beneficial role of the UPR in this process also exists (Thuerauf et al., 2006), since in vitro and in vivo approaches using gain- and loss-of function approaches showed that the UPR protects

cardiomyocytes from cell death. While a detailed understanding of the role of the UPR in ischemic heart disease remains to be established, an emerging concept is that initial low levels of UPR appear to be beneficial and favor adaptive responses, whereas persistent ER stress is detrimental for cell survival.

Reperfusion is the only means to terminate continuous lethal consequences of an ischemic insult. This process, however, comes at a price as reperfusion has been shown to trigger additional damage. Recently, the UPR has been implicated in ischemia reperfusion (IR) (Groenendyk et al., 2010). All available evidence suggests the UPR is strongly activated during IR (Minamino et al., 2010). In cultured cardiomyocytes exposed to conditions simulating IR, significant activation of BiP/GRP78, CHOP, ATF6 and Xbp1s is observed (Ngoh et al., 2009; Yeh et al., 2010). These in vitro observations have been corroborated in vivo in mouse and rat models (Miyazaki et al., 2011; Tao et al., 2011).

Mechanistically, increased ROS levels observed upon reestablishing of oxygen and nutrient supply, as well as rebooting mitochondrial energy production, are likely to stimulate the UPR. Indeed, suppression of ROS production effectively eliminates IR injury and UPR markers are dramatically decreased. Recent evidence suggested that ROS are an integral component in some types of the UPR and contribute there to activating proapoptotic pathways (Santos et al., 2009). UPR inducers can effectively increase ROS formation and knockdown of the proapoptotic branches of the UPR is associated with a drop in ROS levels.

As for the role of the UPR in IR, most studies to date propose models based on the correlation observed using genetic and pharmacological strategies to improve cardiac recovery following IR. While these data provide substantial support for the involvement of the UPR in IR, conclusive results are hard to obtain. The increase in O-GlcNAc modifications can inhibit IR-provoked cardiomyocyte death, which is associated with a decrease in the UPR signaling (Ngoh et al., 2009). Similar results have been obtained using AMPK activators and Apelin (Yeh et al., 2010; Tao et al., 2011). Moreover, recent studies found that IR injury is significantly decreased in mice lacking CHOP (Miyazaki et al., 2011). While these data point toward a detrimental role for UPR in IR injury, other findings lead to alternative conclusions. Natarajan et al. (2009) found that prolyl hydroxylase inhibition can attenuate IR injury, which may be mediated by UPR induction. Acute induction of the UPR by brief exposure to a UPR inducer effectively protects the heart from IR insult (Petrovski et al., 2011). Studies using

an inducible ATF6 overexpression model discovered that UPR induction favors cardiomyocyte survival during IR, by reducing necrosis and apoptosis (Martindale et al., 2006). More studies are warranted to define the biological role of the UPR in IR of the heart.

In summary, despite all the genetic and pharmacological data that seek to establish a direct correlation between IR, ER stress and UPR, a definitive conclusion is still lacking, mainly due to recent results that point to the UPR as an adaptive response under these conditions and not as a cell death pathway per se. Moreover, given the importance of the metabolic changes observed during the development of IR injury, future studies should not only focus on clarifying the metabolic regulation exerted by the ER, but also recognize the importance of this organelle in the control of the cellular homeostasis and how this affects cellular survival.

6.2.2. Cardiac Hypertrophy and Heart Failure

It has been widely reported that both ER stress and UPR are activated in models where pressure or volume overload or mechanical stress are used to induce hypertrophy and heart failure (Okada et al., 2004; Groenendyk et al., 2010; Minamino et al., 2010; Sari et al., 2011). Initially, cardiac hypertrophy is characterized as an adaptive response of the heart to overload. In general terms, if the stress persists, contractile dysfunction ensues and the process is termed *pathological hypertrophy*. However, if contractile function is not affected, the alterations observed are considered as physiological hypertrophy. At the cellular level, hypertrophy is characterized by an increase in the number and organization of sarcomeres, increased protein synthesis and reexpression of genes typically observed in embryonic stages. In addition, all these changes are accompanied by alterations in both calcium homeostasis and metabolism, which, together with increased protein synthesis, can induce ER stress and consequently trigger the UPR (Berridge, 2006; Abel and Doenst, 2011; Dickhout et al., 2011; Ni et al., 2011).

Some studies report that during hypertrophy induced by pressure overload, there is an increase in BiP/GRP78 synthesis and in the activation of the XBP1, IRE1α/TRAF2 pathways (Dickhout et al., 2011; Sari et al., 2011). Alternatively, activation of the fetal gene expression program characteristic of hypertrophy can be altered by ATF6 and XBP-1 by modulating the activation of key transcription factors characteristic of this disease, such as MEF2C, NFAT and GATA (Groenendyk et al., 2010).

Moreover, considering that ATP is required not only for chaperone function but also IRE1 activation, changes in cellular energy metabolism may influence the development and maintenance of the UPR (Burkart et al., 2011). During the onset of cardiac hypertrophy, it is not clear whether changes in fatty acid metabolism and ATP levels occur in cardiomyocytes. However, while studies of physiological hypertrophy suggest an increase in fatty acid metabolism (Burelle et al., 2004; Strøm et al., 2005), this contrasts with the decrease observed during pathological hypertrophy (van Bilsen et al., 2009; Doenst et al., 2010; Abel and Doenst, 2011). However, these changes are still controversial since other studies suggest that during pathological hypertrophy, there are no metabolic changes in cardiac cells (Degens et al., 2006).

Thus, while at the onset cellular metabolism may remain unchanged and UPR may represent part of a cellular adaptation process, persistent stress along with other unknown factors, such as changes in energy levels, could induce a switch from protective ER stress signaling to cell death.

Thus, along with the activation of ER stress, both apoptosis and loss of cardiac function can be observed in the progression from hypertrophy to heart failure. Indeed, ER stress is involved in cardiotoxicity and progression of heart disease to heart failure (Groenendyk et al., 2010; Minamino et al., 2010). Furthermore, cardiac dysfunction is generally associated with a decrease in the cellular energy (Groenendyk et al., 2010; Schulz et al., 2010; Dickhout et al., 2011; Ni et al., 2011).

While various studies report on activation of the UPR in both hypertrophied and failing hearts, activation of proapoptotic CHOP-induced ER stress has only been reported for heart failure or during transition from hypertrophy to failure. Also, for CHOP knockout mice, decreased degrees of hypertrophy and cardiac dysfunction are observed after transverse aortic constriction in comparison to wild-type animals (Okada et al., 2004; Minamino et al., 2010). On the other hand, during pressure overload-induced ER stress, SREBP1c is activated, which in turn induces the synthesis of triglycerides, while the activation of XBP-1 stimulates the synthesis of phosphatidylcholine. These observations, in conjunction with reduced fatty acid oxidation that induces triglyceride accumulation in cardiomyocytes, may contribute to lipotoxicity that has been described in the heart failure (Okada et al., 2004; Groenendyk et al., 2010).

Clearly, further studies are required to clarify the relationship between induction of ER stress in the heart, development of cardiac hypertrophy and progression to heart failure. Similarly, the relationship between UPR

induction and cardiac metabolism is an issue that requires more attention to understand how contractile dysfunction and cell death ensue.

6.3. UPR and Neurodegeneration

In neurons and in several models of neurodegenerative diseases, calcium plays a critical role as a second messenger, controlling synaptic function and cell viability. In basal cellular conditions, ER calcium concentrations are close to 300 μM, whereas cytosolic calcium concentrations usually range between 5 and 50 nM (Pozzan et al., 1994). Several key ER chaperones require optimal calcium concentrations for their protein folding activity, pointing to ER-calcium depletion as an important target in the folding and maturation of proteins and as a key element in the development of some neurodegenerative diseases where the folding capacity of the neurons is challenged (Corbett et al., 1999; Ashby and Tepikin, 2001). Additionally, a sustained increase in the cytosolic calcium may also induce mitochondria-mediated apoptosis via the activation of proteins like calcineurin or by an overload of mitochondrial calcium (Wang et al., 1999). Moreover, recent findings correlate ER stress with the control of mitochondrial function and metabolism through mechanisms involving calcium transfer from the ER to mitochondria (Cárdenas et al., 2010; Troncoso et al., 2011; Bravo et al., 2011a, 2011b). For all these reasons, regulation of the calcium concentration in the cytosol and ER are critical for maintaining normal neuronal function and homeostasis.

One critical example is AD, one of the most common forms of dementia in developed countries, where defects in calcium signaling due to altered ER (specifically MAM) function could explain the well-known disturbances in calcium homeostasis and mitochondrial deficits observed in this disease (Smith et al., 2005; Small, 2009; Yu et al., 2009), including the enhanced IP3-mediated release of calcium in fibroblasts from patients with the sporadic (SAD) (114) or the familial (FAD) form of the Alzheimer disease (Ito et al., 1994; Leissring et al., 1999a, 1999b; Nelson et al., 2007). The majority of FAD cases are caused by point mutations in the genes of two homologous proteins, presenilin 1 (PS1) and presenilin 2 (PS2), essential components of the γ-secretase complex responsible for the production of the amyloid β peptides (Aβ) (Goedert and Spillantini, 2006) that mainly localize to at the MAM surface (Schon and Area-Gomez, 2010). Accumulating evidence suggests that FAD is linked to an imbalance of cellular calcium homeostasis (Bojarski et al., 2008; Mattson, 2010) and, in particular, the presenilins appear to play a key role in the control of calcium concentration within

the ER. In this way, it has been reported that several FAD-linked presenilin mutants altered the expression or sensitivity of the RyR and the IP3R in different models, including cell lines, neurons and brain microsomes (Mattson, 2010). In parallel, it has also been proposed that the SERCA could be a target for presenilins, although opposite regulatory effects have been reported in this respect (Green et al., 2008; Brunello et al., 2009). Finally, Tu et al. reported that WT presenilins form low-conductance calcium leak channels in the ER membrane (Tu et al., 2006; Nelson et al., 2007). These results favor the "calcium overload" hypothesis for FAD, which proposes that the reduced ER calcium leak caused by FAD-linked presenilin mutants results in increased ER-calcium concentrations and exaggerated calcium release upon cell stimulation (LaFerla, 2002; Thinakaran and Sisodia, 2006). However, among all these observations, the most important evidence for a role of ER—and more specifically for MAM—in calcium dysregulation in AD is the finding that PS1 and PS2 are able to directly interact with the IP3R, and that FAD-linked mutants not only dramatically enhance the gating of the channel but also stimulate the production of Aβ (Cheung et al., 2008, 2010). More recently and reinforcing the same concept, Zampese et al. (2011), by directly measuring mitochondrial calcium dynamics, showed that PS2, favors calcium transfer between ER and mitochondria, an effect that was reduced by PS2 downregulation and enhanced by the expression of PS2 mutants. However, how this transfer may modulate mitochondrial function and neuronal metabolism is still a matter of debate.

Since mitochondria need to be strategically positioned at sites where metabolic demand is high (Zenisek and Matthews, 2000; Li et al., 2004), a problem with calcium handling not only would affect the control of the mitochondrial functioning (through an imbalance in the control of the Krebs cycle enzymes) but also would provoke alterations in the calcium-mediated mitochondrial movement along axons, triggering highly deleterious effects on neuronal function in general, and on synaptic transmission in particular (as reported by Stokin et al., 2005; Q. Wang et al., 2008, 2009a; 2009b). According to these data, it has been proposed that a model of altered MAM function could provide a unifying hypothesis to explain many of the features of the AD, including the elevated ratios of $A\beta_{42}/A\beta_{40}$ (via changes in the content of cholesterol), hyperphosphorylation of tau (via changes in the phospholipid metabolism) and aberrant mitochondrial dynamics, with retention of large numbers of fragmented mitochondria in the perinuclear region of cells (via abnormal calcium transport between ER and mitochondria) (Schon and Area-Gomez, 2010).

Notably all these metabolic and morphological features have been found in both FAD and SAD (Wang et al., 2009b), pointing to the MAM hypothesis as a unifying explanation able to relate previously unconnected phenotypes.

Another interesting example is the pathogenesis of prion disease (PrD) where several groups have reported the occurrence of ER stress responses, including the activation of UPR transcription factor XBP1 splicing (Hetz et al., 2008) as well as JNK and ERK (Steele et al., 2007; Hetz et al., 2008). In Creutzfeldt–Jakob disease (CJD) patients and in some mouse models, upregulation of several chaperones and foldases such as BiP/GRP78, GRP94 and GRP58/ERp57 is observed, which suggests abnormal ER homeostasis (Yoo et al., 2002; Hetz et al., 2003, 2005; Brown et al., 2005; Rane et al., 2008). These kinds of perturbations lead to the generation of intermediary misfolded forms of the cellular prion protein (PrPc) increasing its susceptibility to convert into the PrP protease resistant form (PrP^{RES}) in vitro (Apodaca et al., 2006; Orsi et al., 2006). Moreover, recently, Hetz's group described the contribution of calcium as another element in the development of infectious and familial PrD (Torres et al., 2010). Using purified PrP^{RES} from the brain of scrapie-infected mice, they evaluated its impact on ER stress responses and calcium homeostasis. They found that the acute exposure of PrP^{RES} to Neuro2a cells induces release of calcium from the ER and ER stress, associated with the upregulation of several chaperones and foldases including ERp57, BiP/GRP78 and GRP94. Consistent with these results, cells chronically infected with scrapie prions also display altered ER calcium levels. Additionally, scrapie-infected cells are more susceptible to undergo ER stress-mediated cell death, associated with stronger UPR activation after exposure to different ER stress-inducing agents (Torres et al., 2010). The exact mechanisms involved in calcium disturbance and how this directly affects neuronal metabolism remain to be determined.

As a last example, in the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS), motor neurons degenerate with signs of organelle fragmentation, free radical damage, mitochondrial calcium overload, impaired axonal transport and accumulation of proteins in intracellular inclusion bodies (Grosskreutz et al., 2010). In ALS, chronic excitotoxicity mediated by calcium-permeable AMPA-type glutamate receptors seems to initiate a self-perpetuating process of intracellular calcium dysregulation with consecutive ER calcium depletion and mitochondrial calcium overload. In this context, Berridge et al. had postulated a model based on the chronic shift of calcium from the ER compartment to mitochondria, inducing a long-term accumulation of misfolded proteins in the ER due to a chronically low intraluminal

calcium concentration (Berridge, 2002). Calcium seems to be mobilized between the ER and mitochondria in a process called the "ER–mitochondria calcium cycle" (ERMCC), which is driven by the synaptic excitatory input and electrical activity in neurons rather than occurring spontaneously as in nonexcitable cells. Given the hypothetical nature of this model, it can be speculated that reducing ER calcium release may restore balance to the ERMCC, concomitant with a reduction in ER protein throughput and increasing protein-folding fidelity. A different strategy could be to block the mitochondrial uniporter and thereby prevent mitochondrial calcium overload but the effect of this on overall cellular metabolism needs to be revisited.

As we can see from the previous examples, prolonged ER stress and mitochondrial dysfunction is a key feature of a broad range of neurodegenerative diseases, suggesting that disruption of the ER–mitochondrial axis may be responsible for the metabolic alterations detected and associated with these diseases. Future studies should focus in the establishment of a clear relationship between the metabolic regulation exerted by the ER on mitochondria and how this connection might be related to other features of these diseases.

6.4. UPR and Cancer

Elevated proliferation rates of cancer cells observed during tumor development require increased membrane and protein synthesis. These changes pose major challenges to the molecular machinery within and associated with the ER. In this context, steps of adaptation involving an increase in the ability to sustain adequate protein folding are essential. Cell proliferation associated with tumor growth often takes place in hypoxia and conditions of limited substrate availability ("starvation"). As one possibility, tumor cells adapt to this "stressful" environment by activating the UPR and, in this scenario, an adaptive UPR response favors tumor growth. Alternatively, however, a sizable body of literature is also available indicating that enhancing the UPR may represent a mechanism to preclude tumor progression. Both these facets must be taken into account in developing a better understanding of how the UPR participates in oncogenesis and particularly how such insights might be exploited in novel cancer therapies. In this section, we will focus the discussion on such aspects.

6.4.1. UPR in Tumor Cell Survival

The ability to upregulate the UPR appears to be essential for tumor survival (Bi et al., 2005). Indeed, the UPR has been implicated in

inflammation-induced oncogenesis through heat-shock proteins (Goldstein and Li, 2009). Both the ATF6 and IRE1 branches induce BiP/GRP78 expression in conditions of ER stress and this chaperone promotes ER homeostasis and resistance to apoptosis by interacting with BIK and caspase-7 (Rao et al., 2002; Reddy et al., 2003; Fu et al., 2007). Conditional ablation of BiP/GRP78 in prostate abolishes tumorigenesis in a PTEN-null mouse, where the chaperone is critical for neovascularization during tumor growth and metastasis. Also, in a transgene-induced breast cancer model, BiP/GRP78 was shown to favor tumor proliferation and angiogenesis (Fu et al., 2008; Dong et al., 2011). The participation of BiP/GRP78 is also suggested from other studies. In particular, a correlation between BiP/GRP78 expression and cancer progression is observed in a number of other types of cancers, including gastric carcinomas, melanoma, hepatocellular carcinoma and breast cancer (Shuda et al., 2003; Lee et al., 2006; Daneshmand et al., 2007; Dong et al., 2008; Zheng et al., 2008; Zhuang et al., 2009). Such elevated levels of BiP/GRP78 in tumor cells suggest that basal UPR levels are increased and may contribute to decreased apoptosis. Taken together, currently available data indicate that enhancing the UPR and BiP/GRP78 expression favors adaptation to the hypoxic environment and tumor growth under these restrictive conditions. Moreover, essentially as a corollary, recent preclinical studies have reported on the use of BiP/GRP78 inhibitors for cancer treatment (Backer et al., 2009).

6.4.1.1. IRE1-XBP1 Branch

Several lines of evidence suggest that the IRE1-XBP1 branch of the UPR is essential for tumor cell survival under hypoxic conditions and maintenance of malignancy (Romero-Ramirez et al., 2004). For instance, in XBP1-deficient cells, increased apoptosis and decreased clonogenic survival are observed under hypoxic conditions. Upon subcutaneous injection of immunodeficient mice with XBP1 knockout cells, decreased tumor growth and higher sensitivity to hypoxia was observed in comparison to wild-type controls (Romero-Ramirez et al., 2004). Furthermore, downregulation of XBP1 using specific shRNA diminished blood vessel formation in tumors from mouse embryonic fibroblast and fibrosarcoma cells and expression of the spliced form of XBP1 restored angiogenesis in cells expressing dominant negative IRE1α (Romero-Ramirez et al., 2009). The latter observations provide evidence linking successful angiogenesis to the IRE1-XBP1 branch in tumor cells. Moreover, in transgenic mice, expressing an XBP1-luciferase

construct as a reporter for ER stress and downstream responses relevant to the tumor microenvironment, a positive correlation between XBP1-luciferase activity and tumor growth rates was observed for several primary mammary tumor cells (Spiotto et al., 2010). This branch is also implicated in transcriptional regulation of VEGF, an angiogenic factor highly expressed in tumors (Plate et al., 1992) that protects them against ischemic injury and plays a prominent role in sustaining tumor growth (Shweiki et al., 1992). VEGF mRNA increases under conditions of ER stress, but when IRE1 is silenced by shRNA, VEGF induction is decreased, resulting in the loss of blood vessels. These authors also demonstrated that the two other branches (PERK, ATF6) independently regulate VEGF expression and that inactivation of any one of these three pathways impairs VEGF upregulation as a consequence of ER stress, suggesting that coordinated activation of all three pathways is essential for increased VEGF expression (Ghosh et al., 2010).

6.4.1.2. PERK Branch

Activation of the PERK branch is considered an essential part of the responses triggered in tumor cells required for survival in hypoxia. In tumors, PERK is activated, phosphorylates eIF2α and thereby favors activation of ATF4 and NRF2, transcription factors which promote cell survival by increasing autophagy and activating antioxidant mechanisms. Initial evidence from a study in MEF cells expressing an eIF2α mutant (S51A) that cannot be phosphorylated by PERK indicated that survival of such cells under prolonged hypoxia was reduced (Bi et al., 2005). PERK ablation decreased tumor growth rates in nude mice and in PERK knockout mice, reduced insulinoma formation and vascularization were observed (Bi et al., 2005; Gupta et al., 2009). The ATF4 pathway is also implicated in tumor adaptation in the response to hypoxia (Ye et al., 2010). One of these tumor adaptation responses is related to enhanced expression of VEGF, whereby synthesis and processing in the ER for secretion (Ferrara and Davis-Smyth, 1997) are increased in hypoxia by mechanisms involving the ATF4 pathway (Roybal et al., 2004). On the other hand, secretion of de novo synthesized VEGF is controlled by a heat-shock protein 70 family molecular chaperone localized in the ER (ORP150), which is upregulated under hypoxia (Ozawa et al., 2001). In addition, PERK participates in the survival of dormant tumor cells found in small, chemotherapy resistant and asymptomatic tumors, via mechanisms involving augmented expression of BiP/GRP78, reduced protein synthesis and exit from the cell cycle in the G1 phase (Ranganathan et al., 2006). Alternatively, PERK facilitates survival

of extracellular matrix (ECM)-detached cells by promoting autophagy, ATP production and an antioxidant response in human mammary epithelial cells. This mechanism may potentially be exploited by ECM-detached tumor cells in breast ductal carcinoma in situ lesions (Avivar-Valderas et al., 2011). However, paradoxically, PERK ablation was recently also shown to promote mammary tumorigenesis in aged mice through increased ROS and DNA damage. In doing so, PERK ablation favors DNA mutation and neoplastic growth (Bobrovnikova-Marjon et al., 2010).

6.4.1.3. ATF6 Branch

The role of the ATF6 branch in cancer has been poorly explored to date, although indirect evidence exists indicating that ATF6α is an important modulator of chronic ER stress. For instance, a complex between XBP1 and ATF6α that upregulates ERAD components is detected in MEF cells (Yamamoto et al., 2007). This process must be carefully regulated as cells degrade proteins whose synthesis consumes high levels of ATP. Moreover, the ERAD pathway per se also requires large amounts of ATP. A second piece of evidence implicating ATF6α as a key regulator in persistent ER stress was obtained using cells from ATF6α knockout mice. There, reduced survival following long-term ER stress correlated with lower levels of chaperones, such as BiP and GRP94 (Wu et al., 2007). Indirect evidence implicating ATF6 is provided by studies identifying p50ATF6 within the nucleus in moderately or poorly differentiated hepatocellular carcinomas (Shuda et al., 2003). On the other hand, ATF6 activation is crucial to long-term survival of tumors by augmenting resistance to chemotherapy, nutrient starvation and stress in quiescent squamous carcinoma cells, where ATF6 increases Rheb expression, which in turn activates mTOR activity in an Akt-independent manner (Schewe and Aguirre-Ghiso, 2008). Finally, activation of the UPR, through PERK, IRE1α and ATF6, promotes VEGF expression and subsequent initiation of the angiogenic program (Ghosh et al., 2010).

6.4.2. UPR as an Adaptive State in Cancer

Generally, normal, nonsecretory cells are not subject to permanent ER stress and the UPR pathways exist in a quiescent state. In contrast, tumor cells must adapt to hypoxia and a stressful environment, and do so in ways, which require developing an adaptive UPR state. Although the precise molecular signals that induce this state in tumor cells remain poorly defined, high ROS levels and protein synthesis rates, which are prevalent in cancer cells,

are likely to be involved. In normal mammalian cells, considerable evidence is available indicating that excessive ER stress induces the apoptotic program through CHOP and JNK activation (Zinszner et al., 1998; Hatai et al., 2000). Additionally, mitochondrial calcium overload promotes apoptosis mediated by ERO1α and BIM expression (Tabas and Ron, 2011). However, unlike normal cells, tumor cells often become resistant to apoptosis either by increasing the expression of antiapoptotic proteins or by reducing the function of proapoptotic factors. In an analogous manner, tumor cells also modulate their UPR so as to favor proliferation, antioxidant responses, metabolic plasticity and survival in conditions of hypoxia and nutrient starvation. Taken together, these data suggest that during tumor development, cancer cells undergo an adaptive response that facilitates their survival by downregulating components of the UPR response that are utilized to provoke death in normal cells.

6.4.3. ER–Mitochondria Connection

In addition to these ER-localized stimuli, mitochondrial stress induced by calcium, nitric oxide or oligomycin is also known to activate PERK (Lu et al., 2009; Silva et al., 2009; Bollo et al., 2010). Additionally, nitric oxide induces mitochondrial calcium release and activation of calcium-dependent serine protease, which cleaves p90ATF6 to yield p50ATF6. The later translocates to the nucleus and promotes transcription of cytoprotective GRP78 (Xu et al., 2004). Thus, both mitochondrial and ER stress can induce UPR activation. Moreover, it is important to bear in mind that calcium shuttling between ER and mitochondria plays an important role in regulating metabolism, autophagy and apoptosis. Available evidence indicates that as an early response to ER stress, ER–mitochondria coupling, mitochondrial OXPHOS activation and enhanced ATP synthesis are observed (Bravo et al., 2011a, 2011b). Several studies now show that MAMs are involved in the transfer of calcium from ER to mitochondria and proteins specifically localized in this compartment regulate this function. Moreover, loss-of-function mutations of some proteins localized in MAMs promote cell proliferation and desensitize cells to apoptotic stimuli (Chen et al., 2004; Simmen et al., 2005; Hayashi and Su, 2007). Based on such observations, it has been proposed that in tumor cells, the interaction between ER and mitochondria may be affected in a manner that alters the UPR response.

PACS-2 is a protein involved in protein sorting that localizes to MAMs (Simmen et al., 2005). Reduction or depletion of PACS-2 expression has been reported in cancer tissues (Aslan et al., 2009). PACS-2 knockdown

induces BiP/GRP78 expression and reduces the capacity to undergo apoptosis in response to staurosporine, thapsigargin or anti-FAS antibodies (Simmen et al., 2005). Sig-1R is a chaperone protein that is highly expressed in several tumor cell lines (Aydar et al., 2006). Pharmacological agonists of Sig-1R function promote resistance to apoptosis (Spruce et al., 2004). This protein is found in MAMs, where it interacts with BiP/GRP78 (Hayashi and Su, 2007). Upon ER stress, Sig-1R dissociates from BiP/GRP78 and promotes mitochondrial calcium uptake via the inositol triphosphate receptor. Overexpression of Sig-1R counteracts ER stress-induced apoptosis (Hayashi and Su, 2007). This protein may also play an important role in cancer progression. In physiological conditions, Sig-1R induces mitochondrial calcium uptake, while its overexpression results in resistance to apoptotic stimuli (Hayashi and Su, 2007). Mitofusin 2 (Mfn2) is involved in mitochondrial dynamics and fusion of the outer mitochondrial membrane. Mfn2 is localized in MAMs, where it participates in the transfer of calcium between ER and the mitochondria. Mfn2 loss-of-function increases ER and mitochondria calcium levels but decreases mitochondrial calcium uptake (de Brito and Scorrano, 2008). On the other hand, decreased levels of Mfn2 enhance proliferation while its overexpression induces cell cycle arrest in rat vascular smooth muscle cells (Chen et al., 2004).

Available data suggest that ER–mitochondria coupling is essential to regulate proliferation or apoptosis. In addition, proteins involved in ER morphology may also impact ER–mitochondria coupling. A recent study revealed an interesting link between ER stress and mitochondrial adaptation in an animal model of pulmonary arterial hypertension (PAH), a pathology characterized by proliferation and resistance of smooth muscle cells to death signals (Sutendra et al., 2011). These authors further demonstrated that NOGO-B, a reticulon-4 isoform that participates in the formation and maintenance of ER shape, regulates mitochondrial activity in hypoxia. NOGO-B protein levels are increased in smooth muscle cells isolated from PAH patients subjected to hypoxia. NOGO-B upregulation under those conditions is dependent upon ATF6 activity. In addition, NOGO-B upregulation leads to the disruption of ER–mitochondria coupling. This loss causes a decrease in lipid and calcium transfer from the ER to the mitochondria, which impairs mitochondrial OXPHOS, and decreases Krebs cycle intermediates. Under these conditions, HIF-1α is activated and, subsequently, the glycolytic and antiapoptotic programs. This report suggests that ER–mitochondria uncoupling promotes an adaptive metabolic response, similar to the Warburg effect in proliferating cells. Based

on this, one may hypothesize that alterations in the ER–mitochondria coupling are important for adaptive ER responses to stress in cells exposed to hypoxia or starvation, such as those found in tumor cells.

6.4.4. Therapeutic Strategies Based on Suppressing the UPR

Most of the time, ER stress responses are not observed in normal cells, but in tumor cells, the opposite seems to be the case. This difference between normal and tumor cells can be exploited to develop drugs that target the UPR as anticancer agents. In this respect, two approaches have been described: namely either stimulation of accumulation of misfolded proteins in the ER to overcome UPR, or inhibition of adaptive and antiapoptotic pathways (Li et al., 2011). An example within the first group is bortezomib, a proteasome inhibitor that has efficacy in multiple myeloma (Obeng et al., 2006), as well as cytotoxic effects in breast, colorectal, ovarian, pancreatic, lung and oral cancer cells (Sterz et al., 2008). Other chemical agents that cause accumulation of misfolded proteins are the ERAD inhibitors. For instance, Eeyarestatin I, an inhibitor of the ER-associated p97 ATPase required for ERAD (X. Wang et al., 2008), has been shown to be cytotoxic for hepatocellular carcinoma (HepG2) cells (Cross et al., 2009). Given the importance of chaperones in the UPR, the use of agents that target ER chaperones as anticancer drugs, emerges as an interesting possibility. Indeed, the HSP90 inhibitor 17AAG (17-allyamino-17-demethoxygeldanamycin) and radicicol activate all three branches of UPR in human myeloma cells, inhibiting proliferation and increasing the expression of BiP and GRP94 chaperones, thereby promoting caspase-dependent cell death (Davenport et al., 2007). For versipelostatin, a BiP/GRP78 inhibitor, cytotoxicity toward glucose-deprived tumor cells was observed, as well as inhibition of UPR in cell lines derived from solid tumors, including human colon cancer HT29, fibrosarcoma HT1080, and stomach cancer MKN47 cells (Park et al., 2004). Also, inhibiting the adaptive IRE1α/XBP1 pathway has been shown to result in antitumor activity. For example, irestatin an inhibitor of XBP1s transcriptional activity reduces growth and survival of hypoxia-exposed myeloma cells in vitro and in subcutaneous xenografts (Li et al., 2011).

6.4.5. UPR in Tumor Cell Death

Thus far, we have reviewed aspects of the UPR that favor the survival of tumor cells. However, persistent or excessive ER stress is known to trigger cell death, mainly apoptosis, by both mitochondria-dependent and -independent pathways (Rao et al., 2002; Breckenridge et al., 2003).

6.4.5.1. IRE1-XBP1 Branch

The IRE1-XBP1 branch has been suggested to connect to apoptosis by recruiting TRAF2, activating procaspase-4, and subsequently caspase-9 and caspase-3, thereby triggering mitochondria-independent apoptosis (Wu and Kaufman, 2006). Paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride), a widely used herbicide, induces UPR, activating the IRE1/ASK1/JNK axis, leading to apoptosis in neuroblastoma SH-SY5Y cells (Yang et al., 2009).

6.4.5.2. PERK Branch

The PERK branch can be activated during loss of adhesion and regulate cellular fate. Inhibition of PERK signaling by the expression of two dominant negative PERK mutants results in hyperproliferative MCF10A mammary epithelial cells. In the same study, introduction of mutated PERK converted MCF10A cells into tumorigenic cells after implantation in denuded mouse mammary fat pads (Sequeira et al., 2007). Moreover, the PERK branch also regulates angiogenesis in response to hypoxia. Using microarray analysis in K-ras transformed $PERK^{-/-}$ cells, Blais et al. (2006) demonstrated that under hypoxic conditions, several proangiogenesis transcripts are less efficiently translated, resulting in increased apoptosis, nonfunctional, irregularly structures blood vessels and reduced microvessel density. The induction of UPR can sensitize tumor cells to cisplatin-induced death. Cisplatin is widely used to treat solid tumors and it is believed to kill cells by binding to DNA and interfering with repair, thereby activating cell cycle arrest and apoptosis (Pascoe and Roberts, 1974). However, cisplatin also efficiently induces UPR and activates caspase-12 in a nucleus-independent manner in human melanoma and colon cancer (HCY116) cytoplasts. In summary, the proapoptotic effects of cisplatin not related to its ability to bind to DNA but may also be linked to effects on protein folding (Mandic et al., 2003). Additionally, a correlation between UPR and sensitization to DNA-crosslinking agents, such as carboplatin (Yamada et al., 1999), melphalan and BCNU (Chatterjee et al., 1997), has been observed. The mechanisms underlying these observations are not clear but it is tempting to speculate that the protein complexes required for DNA repair might be adversely affected by changes in protein synthesis and degradation when UPR is triggered.

6.4.5.3. ATF6 Branch

During metastasis, tumor cells must detach from the ECM, which frequently leads to anoikis. CHOP, a downstream effector in both the ATF6 and PERK branches, is widely attributed proapoptotic functions (Zinszner

et al., 1998). For instance, CHOP is suggested to upregulate proapoptotic BCL2 family members, like BAK/BAD, while downregulating antiapoptotic BCL2 and increasing reactive oxygen species (ROS), which together favor cytochrome c release from mitochondria and trigger apoptosis (Ma and Hendershot, 2004). ER stress conditions also promote calcium release from the ER to the cytosol, thereby favoring activation of calpains, which then cleave and activate caspase-12 (Nakagawa and Yuan, 2000). Then, caspase-12 in turn cleaves and activates cytosolic caspase-9, triggering apoptosis in a manner independent of cytochrome c release from mitochondria.

In summary, UPR is crucial to malignant cell survival in the cytotoxic environment within a tumor. These conditions activate UPR as an adaptive response that allows tumor cells to persist. For this reason, the possibility of developing chemical agents that target some key UPR components is an interesting therapeutic option. On the another hand, if ER stress conditions prevail and cellular homeostasis cannot be reinstated, UPR can trigger apoptosis. Indeed, as we have detailed here, UPR can sensitize tumor cells to certain anticancer agents. However, given that in tumor cells protein expression and signaling are frequently altered in ways that allow these cells to avoid apoptosis, we suspect that the balance is tilted toward UPR as a prosurvival response. In any case, an integrated vision of the UPR in tumor cells is essential to understand how this response contributes to the cancer growth.

7. CONCLUSION AND PERSPECTIVES

The ER is a fascinating organelle that plays a central role within the cell. This extremely complex and heterogeneous cellular compartment spreads throughout the cytoplasm forming various subdomains with diverse morphology and function. As such, the ER is a highly dynamic structure that modulates organization according to cellular needs and participates in protein synthesis, lipid metabolism, calcium handling, metabolic regulation and signaling. All of the latter processes are essential to the cell, making the ER susceptible to a variety of perturbations. The degree of complexity associated with optimal ER function positions this organelle as the largest stress sensor of the cell and, therefore, a major contributing factor to disease.

Under stress conditions, the ER induces the UPR, a well-known signaling cascade that acts as an important regulator of cellular homeostasis. The UPR generates an integrated genetic response that serves to re-establish homeostasis in the always-varying intracellular or extracellular milieu. Failure to regain homeostasis causes the UPR to activate cell death

pathways. This dual role of the UPR has become an important target of study since failure in its activation has been implicated in the onset of multiple pathologies.

One emerging area of study is the ER's ability to interact with other cellular organelles. Contacts between cellular structures and compartments are important in cellular regulation, especially of metabolism and apoptosis. The dynamic nature of various organelles makes their interface susceptible to be remodeled depending on cellular homeostasis; however, this is a topic that still requires more attention.

The properties of the ER determine the physiology of different organs, allowing them to fulfill their normal functions. Sensing of nutrient levels, continuous calcium signaling, protein aggregation and adaptation to ever-changing environments are processes in which the ER plays a vital role. Despite the advances accomplished in the treatment of diabetes, cardiovascular pathologies, neurodegeneration and cancer, many strategies based on ER function are not yet fully understood and remain to be developed. Additional studies should address the enormous therapeutic opportunities the ER holds.

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CHAPTER SIX

Regulation of Blood–Testis Barrier (BTB) Dynamics during Spermatogenesis via the "Yin" and "Yang" Effects of Mammalian Target of Rapamycin Complex 1 (mTORC1) and mTORC2

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Contents

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Abstract

In mammalian testes, haploid spermatozoa are formed from diploid spermatogonia during spermatogenesis, which is a complicated cellular process. While these cellular events were reported in the 1960s and 1970s, the underlying molecular mechanism(s) that regulates these events remained unexplored until the past ~10 years. For instance, adhesion proteins were shown to be integrated components at the Sertoli cell–cell interface and/or the Sertoli–spermatid interface in the late 1980s. But only until recently, studies have demonstrated that some of the adhesion proteins serve as the platform for signal transduction that regulates cell adhesion. In this chapter, a brief summary and critical discussion are provided on the latest findings regarding these cell-adhesion proteins in the testis and their relationship to spermatogenesis. Moreover, antagonistic effects of two mammalian target of rapamycin (mTOR) complexes, known as mTORC1 and mTORC2, on cell-adhesion function in the testis are discussed. Finally, a hypothetic model is presented to depict how these two mTOR-signaling complexes having the "yin" and "yang" antagonistic effects on the Sertoli cell tight junction (TJ)-permeability barrier can maintain the blood–testis barrier (BTB) integrity during the epithelial cycle while preleptotene spermatocytes are crossing the BTB.

ABBREVIATIONS

AJ adherens junction, also known as zonula adherens
AMPK AMP-activated protein kinase
Arp2/3 complex actin-related protein 2/3 complex
BTB blood–testis barrier
Caco-2 cells human colonic epithelial-2 cells
CAR coxsackievirus and adenovirus receptor
Cx connexin
Deptor DEP domain-containing mTOR-interacting protein, an inhibitor of mTORC1 and mTORC2
Dlg discs large
DS desmosome
4E-BP1 4E-binding protein 1
EC ectodomain module
Eps8 epidermal growth factor pathway substrate 8
ERK1/2 extracellular regulated kinase ½
ES ectoplasmic specialization

FSH follicle stimulating hormone
GJ gap junction
HnRNP heterogeneous ribonucleoprotein
IGF insulin-like growth factor
JAM junctional adhesion molecule
KGF keratinocyte growth factor
Lgl lethal giant larvae
LH luteinizing hormone
mLST8 target of rapamycin complex subunit LST8, also known as mTOR associated protein LST8 homolog
MAGUK membrane-associated guanylate kinase
MDCK cells Madin-Darby canine kidney cells
MMP matrix metalloproteinase
MTOR mammalian target of rapamycin complex
mTORC1 mTOR complex 1
mTORC2 mTOR complex 2
N-WASP neuronal Wiskott-Aldrich syndrome protein
PAR partitioning defective protein
PDK1 3-phosphoinositide-dependent kinase 1
PI3K phosphoinositide 3-kinase
PIKK PI3K-related kinase
PIP2 phosphatidylinositol (4, 5)-bisphosphate
PIP3 phosphatidylinositol-3, 4, 5-triphosphate
PKB protein kinase B also known as Akt
PKC protein kinase C
PKP-2 plakophilin-2
PRAS40 proline-rich Akt-PKB substrate 40 kDa
PTEN phosphatase and tensin homolog on chromosome 10
Raptor regulatory associated protein of mTOR
Rheb Ras-homolog enrich in brain
Rictor rapamycin-insensitive companion of mTOR
rpS6 ribosomal protein S6
RSK p90 ribosomal S6 kinase
S6K S6 protein kinase also known as mTOR/p70, mTOR p70 ribosomal S6 kinase
SGK1 serum- and glucocorticoid-inducible kinase 1
SKAR S6K Aly/REF-like substrate
TJ tight junction, also known as zonula occludens
TOP 5′-terminal oligopyrimidine
TSC1/2 tuberous sclerosis complex ½ containing TSC1 (hamartin) and TSC2 (tuberin)
ZO-1 zonula occludens-1

1. INTRODUCTION

Spermatogenesis takes place in the seminiferous epithelium, which is composed of germ and Sertoli cells with the Sertoli cell serving as the "mother" or the "nursery" cell that supports and nourishes germs cells at different stages of their development (Cheng and Mruk, 2010a; Griswold, 1998; Mruk and

Cheng, 2004). Spermatogenesis is a complex and precisely regulated process that produces spermatozoa (haploid, 1n) from spermatogonia (diploid, 2n). Spermatogenesis is also tightly controlled by the hypothalamic–pituitary–testicular hormonal axis. This axis involves the production of gonadotropin-releasing hormone (GnRH) from the hypothalamus that induces the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland. LH then stimulates the release of testosterone from Leydig cells via steroidogenesis to support Sertoli and germ cell function, which together with FSH that exerts its effects exclusively on Sertoli cells. These hormones together with locally produced hormones (e.g. inhibin, activin), steroids (e.g. estradiol-17β), and paracrine and autocrine factors (e.g. cytokines, fragments of laminins and collagens), thereby maintaining spermatogenesis in a unique microenvironment in the seminiferous epithelium (Carreau and Hess, 2010; Cheng and Mruk, 2012; O'Donnell et al., 2001; Sharpe, 1994; Walker, 2011; Winters and Moore, 2007). During spermatogenesis, a single type A spermatogonium undergoes 10 successive rounds of mitosis to give rise to 1024 primary spermatocytes, which then enter meiosis to produce 4096 spermatids theoretically (Cheng and Mruk, 2012; Ehmcke et al., 2006). Spermatids then undergo maturation via spermiogenesis to form spermatozoa which are to be released into the tubule lumen at spermiation (O'Donnell et al., 2011). However, it is estimated that the efficiency of spermatogenesis is only ~25%, and the majority of germ cells undergo apoptosis, which is regulated by estrogen produced by Leydig cells, Sertoli cells and germ cells (Barratt, 1995; Shaha, 2008; Tegelenbosch and de Rooij, 1993). This is to prevent overwhelming the capacity of Sertoli cells since each Sertoli cell can support ~30–50 developing germ cells (Billig et al., 1995; Weber et al., 1983). During spermatogenesis, the seminiferous epithelium can be organized into 14 stages in rats (stage I–XIV); 12 stages (stage I–XII) in mice and six stages (I–VI) in humans according to the different developmental stages of germ cells, in particular, the association of developing spermatids with Sertoli cells (de Kretser and Kerr, 1988; Hess and de Franca, 2008; Mruk et al., 2008; Parvinen, 1982). Throughout the seminiferous epithelial cycle, germ cells have to traverse the seminiferous epithelium, from the basal to the adluminal (apical) compartment, and finally reach the luminal edge of the seminiferous tubule at spermiation. This timely translocation of germ cells is synchronized with a series of cyclic junctional restructuring events at the Sertoli–Sertoli and Sertoli–germ cell interface (Cheng and Mruk, 2010b, 2012). These events are tightly regulated and precisely coordinated, their disruption can perturb spermatogenesis, leading to infertility.

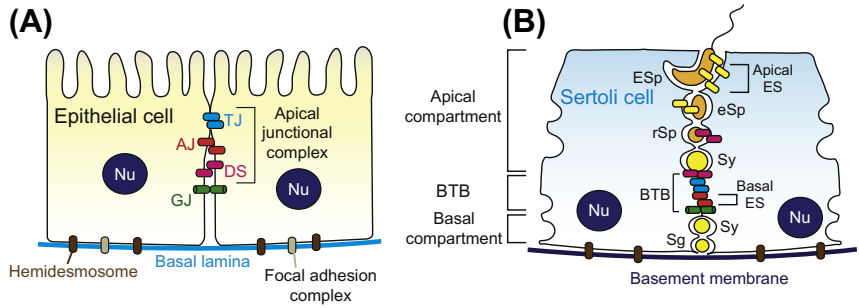

Figure 6.1 ***Differences in the morphological layouts of junction types between a typical epithelium/endothelium and the seminiferous epithelium.*** (A) For the junctional complex in typical epithelia/endothelia, TJs, which are responsible for sealing the intercellular space to create the barrier function by regulating paracellular and transcellular transport, are located at the apical region of the lateral membrane between adjacent epithelial/endothelial cells. Underneath TJs, there are AJs that contribute to most of the adhesive force of the apical junctional complex by connecting to a dense F-actin network, creating the zonula adherens plaque, to be followed by desmosomes. Both TJ and AJ are actin-based cell–cell anchoring junctions, whereas DS is intermediate filament-based cell–cell anchoring junction. Other junctional molecules such as GJs, which are not part of the junctional complex, are localized basal to the junctional complex (constituted by TJ, AJ and DS). (B) Unlike the junctional complex in typical epithelia which are furthest away from the basal lamina, the BTB in seminiferous epithelium is located near the basement membrane (a modified form of extracellular matrix in the testis). Instead of being arranged as discrete structure as in other epithelia/endothelia, TJs, basal ES (a testis-specific actin-rich AJ) and GJs are *coexisting* at the BTB, which together with DS are all involved in creating the BTB. The BTB physically separates the seminiferous epithelium into the basal and apical (adluminal) compartments. Spermatogonia and preleptotene spermatocytes reside at the basal compartment, and preleptotene spermatocytes that arise at stage VII-VIII of the epithelial cycle in the rat testis are the only germ cells that can traverse the BTB. After traversing the BTB, spermatocytes undergo meiosis and eventually differentiate into elongating/elongated spermatids, and spermatids (step 8-19 spermatids in the rat testis) anchored to the Sertoli cells by apical ES. Furthermore, hemidesmosomes (intermediate filament-based cell–matrix anchoring junction) and focal adhesion complexes (FAC, or known as focal contacts, an actin-based cell–matrix anchoring junction) are also found in most epithelia, but FAC is absent in the seminiferous epithelium. Abbreviations used: Sg, spermatogonium; Sy, spermatocyte; rSp, round spermatid; eSp, elongating spermatid; ESp, elongated spermatid; Nu, Sertoli cell nucleus; DS, desmosome; AJ, adherens junction; GJ, gap junction; TJ, tight junction; ES, ectoplasmic specialization. For color version of this figure, the reader is referred to the online version of this book.

During the transit of preleptotene spermatocytes conneced in "clones" via intercellular bridges from the basal to the apical compartment, spermatocytes have first to travel across a blood–tissue junctional barrier, which physically separates the two compartments (Fig. 6.1). This junctional barrier, which located near the basement membrane, is formed by adjacent

Sertoli cells known as the blood–testis barrier (BTB). The BTB is one of the tightest blood–tissue barriers, possibly because it is constituted by *coexisting* tight junction (TJ), basal ectoplasmic specialization [basal ES, a testis-specific adherens junction (AJ)], gap junction (GJ), and desmosome (DS) (Cheng and Mruk, 2012; Wong and Cheng, 2005). Except for DS which utilizes vimentin-based intermediate filaments as the attachment site, the above adhesion junctions are all connected to the actin cytoskeleton, especially the basal ES which possesses tightly packed actin filament bundles that lie perpendicular to the Sertoli cell plasma membrane and are sandwiched between cisternae of endoplasmic reticulum and the opposing Sertoli cell plasma membranes. This is also the hallmark ultrastructure of the BTB, which contributes to the unusual adhesive strength of the barrier (Cheng and Mruk, 2010b, 2011; Mruk et al., 2008). Despite the unusual tightness of the BTB, it undergoes cyclic restructuring during stage VIII–XI of the epithelial cycle to facilitate the transit of preleptotene spermatocytes at the BTB by assembling "new" BTB behind the transiting spermatocytes while the "old" BTB above the spermatocytes is being disassembled, so that the immunological barrier can remain intact (Cheng and Mruk, 2011; Cheng et al., 2010). Thus, the BTB serves as an immunological barrier to "seal" developing spermatocytes and spermatids from the systemic circulation, preventing the development of immune responses against germ cells residing at the apical compartment which arise at puberty (Fijak et al., 2011; Meinhardt and Hedger, 2011). This hypothesis about the coexistence of an "old" and a "new" BTB that enclose the spermatocytes in transit at the BTB was designated the intermediate compartment (Russell, 1977), and was shown in a lanthanum study using electron microscopy from our laboratory (Yan et al., 2008c) (Fig. 6.2). As different types of junctions at the BTB are connected to the actin cytoskeleton, BTB restructuring can be effectively regulated via cyclic reorganization of F-actin network utilizing different actin-regulating proteins. These actin-regulating proteins include epidermal growth factor pathway substrate 8 (Eps8) (Lie et al., 2009), which is an actin barbed-end capping and bundling protein (Hertzog et al., 2010), and actin-related protein 3 (Arp3) (Lie et al., 2010), which together with Arp2 forms the Arp2/3 complex that induces branched actin polymerization (Goley and Welch, 2006). Besides, accumulating evidence suggests that mammalian target of rapamycin (mTOR), a signaling molecule and a nonreceptor protein Ser/Thr kinase that is known to modulate an array of cellular events (Weichhart, 2012; Zoncu et al., 2011), is also responsible for the extensive reorganization of F-actin network to assist BTB restructuring during the epithelial cycle of spermatogenesis (Mok et al., 2012a; Mok et al., 2012c).

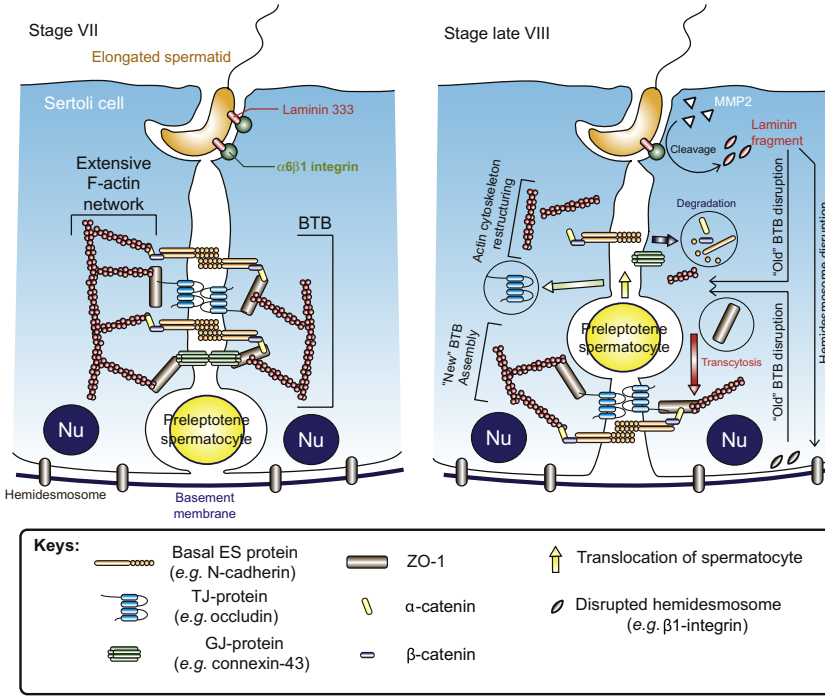

Figure 6.2 ***Restructuring of the BTB to facilitate the transit of preleptotene spermatocytes at stage VIII of the epithelial cycle.*** Before BTB restructuring takes place, its integrity is maintained by *coexisting* TJs, basal ES and GJs which interact with each other and linked to actin cytoskeleton for structural support via adaptor proteins such as ZO-1. Besides, desmosome is also present at the Sertoli cell–cell interface at the BTB. On the other hand, elongated spermatids are also anchored to the Sertoli cell via a testis-specific apical ES protein complex in which laminin-333 residing at the elongating spermatid is linked to α6β1-integrin restricted to the Sertoli cell. At stage VIII of the epithelial cycle, when preleptotene spermatocytes are in transit at the BTB to enter the apical compartment for further development, the "old" BTB above the spermatocyte disassembles to "open" the BTB. This process is mediated by the apical ES–BTB–hemidesmosome functional axis, in which laminin 333 at the apical ES is cleaved by MMP2 to generate bioactive laminin fragments. The laminin fragments induce disruption of the "old" BTB and cause the loss of hemidesmosome function which also contributes to the "opening" of the "old" BTB. Besides, BTB restructuring is also facilitated by mTORC1 as well as by the reorganization of actin cytoskeleton mediated by actin-regulating proteins, such as the Arp2/3–N-WASP complex and Eps8 which induce a "branched/debundled" and "bundled" configuration of the actin filaments at the basal ES, respectively. Without the support from the dense F-actin network, BTB proteins are internalized through endocytosis and the internalized BTB proteins can either undergo degradation or being recycled for the assembly of "new" BTB via transcytosis at the base of the preleptotene spermatocytes. It is likely that molecules, such as testosterone, that promote BTB integrity may be working in concert with mTORC2 underneath the spermatocyte in transit to *assemble* a "new" BTB before the "old" BTB above the transiting spermatocyte is *disassembled*, so that the barrier function can remain intact during germ cell movement at the site. For color version of this figure, the reader is referred to the online version of this book.

In this review, we focus on the biology and regulation of the BTB, in particular, the involvement of the two mTOR signal complexes, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), in regulating the intriguing dynamics of the BTB during the epithelial cycle. Studies on mTOR have largely been focused on the role of mTOR as a key modulator of cell survival, particularly in cancer biology (Khokhar et al., 2011; Wander et al., 2011), since mTOR plays a central role in regulating protein synthesis for cell growth, cell proliferation and survival (Howell and Manning, 2011; Senqupta et al., 2010). However, recent studies have shown that mTOR also takes part in a variety of cellular events including actin cytoskeleton reorganization, aging, autophagy, immune responses and barrier function (Inoki et al., 2011; Mok et al., 2012a; Mok et al., 2012c; Oh and Jacinto, 2011; Vassiliadis et al., 2011; Weichhart, 2012). Studies have shown that in podocytes, which are the cells that establish the blood–urine barrier in the kidney, a disruption of the mTOR signaling perturbs the barrier function as a result of internalization of the TJ-adaptor protein ZO-1 (Shorning et al., 2011) and reduced expression of slit diaphragm proteins (proteins which are essential for cell–cell contact and hence barrier function in podocytes) (Vollenbroker et al., 2009). More important, the involvement of mTOR in the BTB modulation via reorganization of actin cytoskeleton has been demonstrated in studies using RNAi to silence rpS6, a downstream signaling molecule of mTORC1 (Meyuhas, 2008), since its knockdown was found to promote TJ-barrier function (Mok et al., 2012c). On the other hand, the knockdown of rictor, a binding partner of mTORC2 (Sarbassov et al., 2004), was shown to disrupt BTB function (Mok et al., 2012a), illustrating the antagonistic effects of these two mTOR complexes on BTB dynamics. In order to have a better understanding of how the BTB is regulated by mTOR, we first provide an update on the latest status of research on the different junction types and the constituent adhesion proteins at the BTB, and how they interact with each other to maintain the barrier homeostasis. We then provide a brief background on mTOR such as the components of the two mTOR signaling complexes and their functions. Finally, we will examine some recent findings regarding the "yin" and "yang" of mTORs on BTB dynamics via the differential actions of mTORC1 and mTORC2 on BTB function.

2. ACTIN-BASED CELL JUNCTIONS AT BTB

Among all the blood–tissue barriers, such as the blood–brain barrier and the blood–urine barrier which are created between neighboring endothelial cells, cell junctions are typically arranged in which TJs are localized at the apical

region, to be followed by discrete AJs and DS, which constitute the junctional complex (Fig. 6.1). In addition, GJs are located basal to the junctional complex (Hartsock and Nelson, 2008; Miyoshi and Takai, 2008) (Fig. 6.1). In these blood–tissue barriers, the permeability barrier is created almost exclusively by TJs which seal the intercellular space between adjacent membranes and confer cell polarity to restrict paracellular and transcellular transport of substances (Steed et al., 2010; Tsukita et al., 2001), whereas AJs which connect to a dense actin filament network confer the adhesion property (Harris and Tepass, 2010). Thus, the *coexisting* TJs, basal ES and GJs which contribute to the barrier and adhesion function of the BTB as an entity is in fact a unique feature amongst all the blood–tissue barriers (Fig. 6.1). Since TJs, basal ES and GJs are all linked to underlying actin cytoskeleton via corresponding adaptors, changes in the organization of actin filaments at the BTB during the epithelial cycle play a significant role in its restructuring. In this section, we briefly discuss each junction type at the BTB and how these junctions associate with the underlying F-actin cytoskeleton, interacting with each other.

2.1. Tight Junction

TJs appear as "kisses" between adjacent epithelial or endothelial cells under electron microscope where two plasma membranes fuse together as illustrated in the Sertoli cell BTB (Cheng and Mruk, 2010b; Steed et al., 2010; Tsukita et al., 2001). In other blood–tissue barriers, TJs are located apically in an epithelium or endothelium and act as "fences" that divide the membranes into apical and basolateral domains. Since integral membrane proteins are freely diffusible in plasma membrane, this "fence" function of the TJ restricts proteins to their respective apical or basal location (Steed et al., 2010; Tsukita et al., 2001), generating apicobasal polarity in an epithelium and to prevent transcellular transport of substances across the barrier. Although the intercellular space is sealed by TJs in which the TJ strands from two neighboring plasma membranes associate laterally with each other to form a "gate," selected ions and/or solutes can pass through these "gates" via paracellular transport, which is dependent on their charge and size (Steed et al., 2010; Tsukita et al., 2001). This "gate" function of TJs varies among cell types due to the differences in the relative proportions of different TJ proteins (Steed et al., 2010; Tsukita et al., 2001). In addition, differences in TJ-strand density also affect permeability of the TJ (Steed et al., 2010; Tsukita et al., 2001). While the "fence" and "gate" functions imply TJs are considerably rigid in nature, TJs are actually dynamic ultrastructures by adjusting their permeability barrier function in response to changes in environment and/or physiological

needs, such as development, cell migration and cell/tissue homeostasis (Steed et al., 2010; Tsukita et al., 2001). This flexibility of TJ is particularly important for the BTB, which undergoes cyclic restructuring to facilitate the transit of preleptotene spermatocytes while its integrity must be maintained to ensure proper development of spermatids via spermiogenesis behind the barrier. Furthermore, TJs are connected to actin cytoskeleton via adaptor proteins, which include zonula occludens-1, -2 and -3 (ZO-1, -2, -3). To date, many TJ proteins have been identified at the BTB, which include claudins, occludin, junctional adhesion molecules (JAMs), tricellulin and coxsackievirus and adenovirus receptor (CAR) (Cheng and Mruk, 2010b; Steed et al., 2010; Tsukita et al., 2001). Among these, claudins, occludin and JAMs are the best-studied TJ proteins at the BTB, which are briefly reviewed herein.

2.1.1. Claudins

Claudins are a family of TJ proteins, each has four transmembrane domains, two extracellular loops and a short cytoplasmic tail (Elkouby-Naor and Ben-Yosef, 2010). To date, 24 members of claudins have been identified (Elkouby-Naor and Ben-Yosef, 2010). Among these, claudin-1 through -8 and -11 have been identified by northerns in rodent testes (Furuse et al., 1998; Morita et al., 1999a, 1999b), whereas claudin 10, 12, and 23 were detected by microarray analysis using mRNAs from rodent testes (Singh et al., 2009). It is generally accepted that claudins are the backbone of TJ strands and are responsible for recruiting other TJ proteins, such as occludin to TJs. Forced expression of exogenous claudins in fibroblasts was able to induce cell adhesion activity by forming networks of TJ-strand-like ultrastructure at cell–cell contacts (Furuse et al., 1998; Kubota et al., 1999). Besides, the importance of claudins as the core structural component of TJs is demonstrated by the inability of forming an intact barrier in mice with specific claudin knockout. For example, mice lacking claudin 1 died shortly after birth due to dehydration as a result of failure in epidermal barrier function (Furuse et al., 2002). Deletion of claudin 5 in mice led to neonatal death, within 10 h after birth because of the absence of the blood–brain barrier (Nitta et al., 2003). Furthermore, knockout of claudin 18 in mice led to disruption of permeability barrier of gastric epithelia, causing paracellular H^+ leakage that results in atrophic gastric epithelia (Hayashi et al., 2012). Knockout of claudin-11, which is expressed specifically in oligodendrocytes and Sertoli cells, led to infertility in mice due to the lack of BTB without TJ strands formed between Sertoli cells (Gow et al., 1999). Besides being the essential building block of TJs, claudins also determine the properties of TJ barriers by assembling TJs with different claudin members.

For example, TJ strands formed by claudin-1 are highly branched network while claudin-11-based TJ strands, as those found in Sertoli cells, are mostly parallel strands with little branching (Gow et al., 1999; Morita et al., 1999b). Furthermore, the selectivity of ions and solutes of a permeability barrier is also dependent on the composition of claudins as illustrated by gain-or-loss function studies in animals, humans or cell lines involving specific claudins. For instance, overexpression of claudin-2, but not claudin-3, in MDCK I cells which express only claudin-1 and -4, leads to a "leaky" TJ barrier, as shown by a decrease in transepithelial electrical resistance (TER) across the cell epithelium. This thus reflects the differential ability among different claudins in conferring the TJ-barrier function (Furuse et al., 2001). Furthermore, in claudin-15 knockout mice, the small intestine displayed malabsorption of glucose due to a disruption of paracellular transport of Na^+ ions across the TJ barrier (Tamura et al., 2011). Claudin-16, however, was shown to be important to paracellular transport of Mg^{2+} across the TJ barrier (Simon et al., 1999).

Claudins also play an important role in maintaining the BTB function during spermatogenesis. In fact, TJ strands at the BTB is contributed significantly by claudin-11 since deletion of claudin-11 leads to a loss of the BTB ultrastructure, resulting in the lack of TJ strands between Sertoli cells (Gow et al., 1999). Interestingly, Sertoli cells, which normally cease to divide after postnatal day 15, are found to be proliferating in adult claudin-11 knockout mice (Gow et al., 1999). This is probably due to the loss of contact inhibition after the disappearance of TJs. This thus suggests that the permeability barrier imposed by claudin-11 also has a role in regulating cell cycle function in Sertoli cells. Furthermore, a recent report has shown that claudin-3 may be a crucial protein involving in the intermediate compartment during translocation of spermatocytes across the BTB (Komljenovic et al., 2009). Immunofluorescence staining illustrated that during the transit of preleptotene spermatocytes across the BTB at stage VII–IX in mice, localization of claudin-3 at the BTB was found apically to preleptotene spermatocytes ("old" BTB) at stage VII; however, at stage VIII–early IX, claudin-3 was detected at both apically ("old" BTB) and basally ("new" BTB) of the translocating spermatocytes; and finally claudin-3 was detected only at the basal side ("new" BTB) of leptotene spermatocytes transformed from preleptotene spermatocytes (Komljenovic et al., 2009). Despite this stage-specific localization of claudin-3 coinciding with the intermediate compartment, this observation requires further verification by functional studies, such as if its knockdown would indeed impede the migration of spermatocytes at the BTB. Additionally, the role of claudin-3 may be species-specific since

claudin-3 is not found at the BTB in the rat testis (Kaitu'u-Lino et al., 2007). Thus, much work is needed to define the role(s) of different claudin(s) in the cyclic restructuring events of the BTB during spermatogenesis.

2.1.2. Occludin

Occludin is the first integral membrane protein identified at the TJ (Furuse et al., 1993). Although occludin shares a similar topography with claudins by having four transmembrane domains, two extracellular loops and a cytoplasmic tail, there is no sequence homology between the two TJ proteins (Cummins, 2012; Furuse et al., 1998). Unlike claudins, which are composed of multiple members in the claudin gene family, no occludin-related gene has been identified thus far, but two occludin isoforms are produced by alternative splicing. Also, unlike claudins, occludin has a relative long cytoplasmic tail. Ser and Thr residues of its cytoplasmic tail are heavily phosphorylated; and studies have shown that phosphorylations at these sites via protein kinases are essential for regulating occludin localization and distribution in epithelia/endothelia. For instance, a study using primary Sertoli cell cultures in vitro has demonstrated that focal adhesion kinase (FAK) is structurally associated with occludin and it also regulates the structural interaction between occludin and ZO-1 (Siu et al., 2009a, 2009b). Furthermore, a knockdown of FAK in Sertoli cells led to a decrease in phosphorylation of Ser and Tyr, but not Thr in occludin, which, in turn, probably resulted in an increase in the internalization of occludin, thereby perturbing the TJ barrier (Siu et al., 2009a). Besides FAK, c-Yes is another nonreceptor protein tyrosine kinase known to be structurally associated with occludin at the Sertoli cell BTB (Xiao et al., 2011). When the intrinsic activity of c-Yes in Sertoli cells with an established functional TJ-permeability barrier that mimicked the BTB in vivo was inhibited by SU6656, a selective c-Yes inhibitor, redistribution of occludin from cell–cell interface to cell cytosol was found, contributing to the disruption of the Sertoli cell TJ barrier (Xiao et al., 2011). Besides FAK and c-Yes, protein kinase C (PKC) also plays a role in modulating the localization of occludin at TJs via its effects to confer the phosphorylation status in occludin. Study reported that upon stimulation of PKC by phorbol 12-myristate 13-acetate (PMA) and 1,2 dioctanoylglycerol (DiC8), phosphorylation of occludin was induced, leading to an increase in occludin localization at the cell–cell interface (Andreeva et al., 2001). The importance of occludin in spermatogenesis was also addressed by studies using synthetic occludin peptide. It was demonstrated that when occludin–occludin interaction between adjacent Sertoli cells was disrupted

via intratesticular injection of peptide corresponding to a segment of the second extracellular loop of occludin, the BTB was compromised, leading to germ cell loss from the epithelium (Chung et al., 2001). Interestingly, when occludin was deleted, occludin knockout mice remained fertile by age 6 weeks at the time the first wave of spermatogenesis occurred (Saitou et al., 2000). However, these occludin knockout mice were found to be infertile by ~40–60 weeks of age, with their seminiferous tubules displayed atrophy and devoid of germ cells (Saitou et al., 2000). Subsequent studies by generating another genetic model of occludin knockdown confirmed that fertility was retained in these mice only from ~6–10 weeks of age (Takehashi et al., 2007), but all occludin knockout mice were infertile by 36–60 weeks of age with the tubules devoid of spermatocytes and spermatids (Saitou et al., 2000; Takehashi et al., 2007). Collectively, these findings illustrate that while other TJ proteins, such as claudins and JAMs, may be able to supersede the loss of occludin at the BTB to maintain spermatogenesis; however, occludin is absolutely essential to maintain the BTB function and spermatogenesis beyond 10 weeks of age in rodents during adulthood, illustrating the functional relationship between BTB and maintenance of spermatogenesis.

Interestingly, the necessity of occludin to spermatogenesis does not apply to humans as occludin was not found in human Sertoli cells in an earlier study (Moroi et al., 1998). However, a recent study by RT-PCR has identified occludin in human Sertoli cells (Xiao and Cheng, unpublished observations), illustrating further study on the function of occludin in huamn BTB is warranted. The lack of occludin in human seminiferous epithelium also illustrates that the BTB is a complex ultrastructure and its constituency is species-specific. Other studies have also shown that the role of occludin in blood–tissue barriers is organ- and/or tissue-specific. For instance, occludin is not essential for the formation of TJ strands; and in some cell types, it is not even needed for the maintenance of TJs. It was reported that occludin was not found in the TJ strands between porcine aortic endothelial cells (Hirase et al., 1997), revealing that in some tissues, occludin is not a constituent protein of the TJ barrier. Moreover, in occludin knockout mice, the TJ barrier formed between intestinal epithelial cells was indistinguishable from those of the wild type ultrastructurally (Saitou et al., 2000), demonstrating that in some epithelia that normally express occludin, a missing of occludin does not necessarily affect the formation and/or maintenance of the TJ barrier. Furthermore, although studies have shown that treatment of synthetic occludin peptide disrupted TJ barrier between Sertoli cells (Chung et al., 2001) as well as that between intestinal epithelial cells (Nusrat et al., 2005), a study in human intestinal T84 epithelial

(T84) cell cultures has shown that the occludin peptide-induced TJ-barrier disruption was mediated by redistribution of other TJ proteins (e.g. claudin-1) and TJ adaptor (e.g. ZO-1) (Nusrat et al., 2005), illustrating occludin may act as a "signaling" regulatory TJ protein. More important, the use of monoclonal antibody against the second extracellular loop of occludin in T84 cells was found to disrupt epithelial cell polarity but not the TJ barrier (Tokunaga et al., 2007). Collectively, these findings illustrate the complex functional role of occludin at the TJ barrier, supporting the notion of its species- and/or tissue-specific function regarding its involvement in TJ-barrier formation and maintenance. Nonetheless, these findings illustrate that occludin, unlike claudins, may have other role(s) and serving as a signaling molecule in controlling the permeability in TJs, such as fine-tuning the barrier function, besides serving as the building block of TJs in some epithelia. This notion is also supported by studies illustrating that overexpression of exogenous occludin in fibroblasts was able to induce the formation of TJ strands, but these TJ strands were shorter and lesser in quantity when compared to those claudin-based TJ strands; and when fibroblasts were co-transfected with occludin and claudins, occludin was recruited to the TJ strands formed by claudins, and together they formed continuous belt-like ultrastructures at the cell–cell interface, which was in contrast to the punctuate pattern when occludin was overexpressed alone (Furuse et al., 1998). More important, while young adult occludin knockout mice at 6–10 weeks of age were fertile but when these mice reached adulthood by >30 weeks, besides being infertile with seminiferous tubules were found to be devoid of spermatocytes and spermatids, calcification in the brain, and chronic gastritis in the gastric epithelium were detected (Saitou et al., 2000), illustrating occludin, and perhaps TJs, may be playing more important cellular roles besides serving as an indispensable protein at the TJ barrier. In this context, it is of interest to note that studies have reported internalization of occludin by caveolae and/or clathrin-mediated endocytosis (Murakami et al., 2009; Schwarz et al., 2007; Shen and Turner, 2005), including the Sertoli cell TJ barrier (Wong et al., 2009; Yan et al., 2008c), illustrating occludin can be rapidly mobilized to other cellular domains to exert its function besides the TJ barrier.

2.1.3. Junctional Adhesion Molecules

JAMs are members of the immunoglobulin superfamily (IgSF) proteins; the extracellular region of these TJ-integral membrane proteins possess two Ig-like domains. Based on sequence homology, JAM family is composed of two subfamilies with one of them comprises three closely related members namely JAM-A (JAM-1), JAM-B (JAM-2) and JAM-C (JAM-3). Another subfamily,

in which the members have a lower polypeptide sequence similarity, includes CAR, JAM-D (JAM-4) and JAM-like (JAM-L). Herein, we focus on the former subfamily since its members have been better characterized and studied in the testis. JAMs differ from claudins and occludin topologically since each JAM molecule has only one extracellular domain, a single transmembrane region and a cytoplasmic tail that varies in length among different isoforms (Mandell and Parkos, 2005; Severson and Parkos, 2009). Unlike claudins and occludin, JAMs alone is insufficient to from TJ strands as no TJs were detected in many primary cultures of fibroblasts and established fibroblast cell lines that expressed either JAM-A or JAM-C. However, JAMs are concentrated to the TJs when examined by immunofluorescence microscopy (Morris et al., 2006). JAMs are also distributed in and around TJ strands under electron microscopy, indicating their intimate association with the TJ barrier (Itoh et al., 2001). The involvement of JAM proteins in TJ-barrier function has been revealed in several studies. For instance, a study in T84 human intestinal epithelial cells using anti-JAM-A antibody has shown that JAM-A is necessary for recovery of Ca^{2+} depletion-induced TJ-barrier disruption as re-establishment of TJ barrier was disrupted due to the loss of JAM-A and occludin function following antibody treatment (Liu et al., 2000). JAMs are also required for the resealing of a disrupted TJ barrier induced by treatment of epithelial cells with synthetic peptides corresponding to the extracellular domain of JAMs (Liang et al., 2000). Moreover, a leaky TJ-permeability barrier was found in the intestinal epithelial cells of JAM-A knockout mice, indicating the significance of JAM proteins in barrier function (Laukoetter et al., 2007). Interestingly, such leaky TJ barrier might be the result of an induction of claudin-10 and -15 detected in the intestinal epithelial cells obtained from JAM-A knockout mice *versus* the wild-type. It was shown that an induction of certain claudins would lead to an increase in permeability of certain ions across the TJ barrier (Laukoetter et al., 2007). An induction of claudins after knockout of JAM-A and a down-regulation of occludin after JAM-A antibody treatment thus illustrate that JAMs may regulate the TJ barrier by altering the localization and/or expression of other TJ proteins (Severson and Parkos, 2009). Regardless of the importance of JAMs in modulating the barrier function in cell lines or intestinal epithelia, the significance of JAMs to the BTB remains unknown. Although JAM-A and JAM-B are found in the BTB (Morrow et al., 2010), deletion of JAM-A or homozygous mutation of JAM-B had no impact on the BTB integrity (Sakaguchi et al., 2006; Shao et al., 2008). It is known that mice with JAM-A deleted or JAM-B mutated remained fertile and their seminiferous epithelium was histologically normal (Sakaguchi et al., 2006; Shao et al.,

2008). Even though deletion of JAM-A in mice led to reduced litter size, this is probably resulted from impaired motility of spermatozoa as JAM-A was also shown to be involved in sperm tail formation (Shao et al., 2008).

Unlike claudins and occludin whose functions are mostly related to the TJ-permeability barrier as these are structural components of the blood-tissue barriers, JAMs are involved in numerous cellular functions and pathological conditions, such as leukocyte migration, angiogenesis, hypertension and tumorigenesis (Bazzoni, 2011). Among them, the participation of JAMs in the transmigration of leukocyte across the endothelial TJ barrier during inflammation is of great interest since preleptotene spermatocytes may be utilizing JAMs to traverse the BTB with similar mechanism (Wang and Cheng, 2007). It is noted that besides Sertoli cells, germ cells also expressed JAM proteins including JAM-A and JAM-C (Wang and Cheng, 2007), thus it was proposed that other than playing the role for anchoring germ cells to Sertoli cells, JAMs may also be responsible for the spermatocyte transit at the BTB. In fact, the loss of JAM-C, an integrated component of the apical ES at the Sertoli–spermatid interface, led to failure of spermiogenesis and infertility (Gliki et al., 2004). In short, much work is needed to define the role of JAMs during spermatogenesis, in particular, its function at the BTB.

2.1.4. ZO Adaptor Proteins

Underneath the TJs, cytoplasmic plaques are formed via the cytoplasmic tails of TJ proteins directly associated with adaptor proteins, such as ZO proteins, at a 1:1 stoichiometric ratio (e.g. occludin-ZO-1, claudin-ZO-1, JAM-ZO-1), which in turn bind to the underlying actin filaments. As such, TJ proteins are linked to actin cytoskeleton for the support of barrier integrity. Three ZO proteins have been identified thus far and they are ZO-1, ZO-2 and ZO-3, which share sequence homology with each other and among them, ZO-1 is the predominant adaptor protein (Gonzalez-Mariscal et al., 2000; Tsukita et al., 2009). ZO proteins belong to the membrane-associated guanylate kinase (MAGUK) family, and beginning from their N-terminal region, they all have three PDZ domains, to be followed by an SH3 domain, a GUK domain and a cytoplasmic tail. The first PDZ domain was shown to bind to claudins (Itoh et al., 1999a) while the second one is necessary for homo- or heterodimerization between ZO proteins (Utepbergenov et al., 2006; Wittchen et al., 1999), and the third PDZ domain is needed for interacting with JAMs (Bazzoni et al., 2000; Ebnet et al., 2000). ZO proteins associate with occludin using the GUK domain (Furuse et al., 1994; Haskins et al., 1998; Itoh et al., 1999b), with actin filaments link to the ZO proteins

via their cytoplasmic tails (Fanning et al., 1998; Itoh et al., 1997). The knockout of ZO-1 or ZO-2 in mice results in embryonic lethality (Katsuno et al., 2008; Xu et al., 2008). This demonstrates these two ZO proteins are essential for development, but little information can be deduced for their physiological function from these knockout mice. The importance of ZO proteins in recruitment of TJ proteins, especially claudins for the formation of TJs, was revealed by cultured epithelial cell line without endogenous ZO-3, whereas ZO-1 was knockout by homologous recombination, and ZO-2 was knockdown by RNAi (Umeda et al., 2006). Interestingly, when ZO truncated proteins containing only the N-terminus which has the three PDZ domains were forcibly localized to lateral membrane and dimerized, TJs formed by claudins were found to be distributed throughout the lateral membrane (Umeda et al., 2006). This is in sharp contrast to the TJs formed by overexpressing full length ZO-1 and ZO-2 as these TJs are precisely localized to the apical junctional complex. These observations thus illustrate that the interaction of SH3 domain and GUK domain in ZO proteins with AJs is necessary for directing TJ proteins to their correct cellular location (Umeda et al., 2006). The importance of ZO proteins in spermatogenesis was demonstrated in a study by injecting ZO-$2^{-/-}$ embryonic stem cells into wild-type blastocysts to generate viable ZO-2-deficient mice, these mice were found to have reduced fertility, resulting from impaired spermatogenesis, because the BTB was disrupted due to mislocalization of integral membrane proteins claudin 11 and Cx43 at the site (Xu et al., 2009).

These studies thus illustrate the significance of ZO-adaptor proteins in maintaining the TJ-barrier integrity by proper localization of TJ (and also GJ) integral membrane proteins, and this is mediated by their connection to the underlying actin filaments. This is particularly important for the BTB where an extensive F-actin network is present, and is under cyclic restructuring during the epithelial cycle of spermatogenesis. The maintenance and modulation of barrier function by actin reorganization is demonstrated in numerous studies. For instance, when actin depolymerization was induced by latrunculin A (Lat A, a toxin produced by red sea sponge) in MDCK cells, a disruption of the TJ barrier was detected, which was attributed to the internalization of occludin that caused by the loss of the apical perijunctional F-actin ring underneath the TJs, illustrating the importance of actin cytoskeleton for proper TJ-protein localization (Shen and Turner, 2005). In a study using rat alveolar epithelial cells, strengthening of cortical actin filaments induced by treatment of keratinocyte growth factor (KGF) led to a tightening of the TJ barrier (LaFemina et al., 2010). The necessity of an

actin cytoskeleton for the maintenance of the BTB integrity is best illustrated in studies using actin regulating proteins Eps8 and Arp3 (Lie et al., 2010, 2009). It was reported that after in vitro knockdown of Eps8 in Sertoli cells with an established functional TJ-permeability barrier by RNAi, actin disorganization was detected, leading to the redistribution of occludin and ZO-1 from the cell–cell interface into the cell cytosol (Lie et al., 2009). Moreover, in vivo knockdown of Eps8 in testis also led to truncation and mislocalization of F-actin and occludin, respectively, contributing to the disruption of the BTB integrity when assessed by an in vivo BTB functional assay (Lie et al., 2009). Furthermore, in a study using wiskostatin to block Arp3 activation in cultured Sertoli cells, the inhibition of branched actin polymerization that resulted in deposition of actin filament bundles at the cell–cell interface, led to a promotion of the Sertoli cell TJ-permeability barrier function (Lie et al., 2010). Indeed, one of the most important findings from the above studies was that it illustrated the two actin regulating proteins Eps8 and Arp3 that exhibited stage-specific and restrictive spatiotemporal expression at the BTB during the seminiferous epithelial cycle provided the means for cyclic reorganization of the actin cytoskeleton at the Sertoli cell BTB (Lie et al., 2010, 2009). In fact, besides binding to AJs, TJs and actin, adaptor proteins ZO-1/2/3 also bind to GJs, polarity proteins (e.g. PATJ), actin-binding proteins (e.g. cortactin, AF-6) and a variety of signaling molecules, such as kinases (e.g. c-Src, PKC), transcription factors (e.g. ZONAB, c-Jun) and G proteins (e.g. G protein α subunit) (Gonzalez-Mariscal et al., 2000; Tsukita et al., 2009). Thus, these adaptor proteins also act as scaffolding proteins at the TJ barrier by recruiting other regulatory proteins to the site and to provide cross talks among coexisting junctions at the BTB including TJs, basal ES and GJs.

2.2. Ectoplasmic Specialization (ES)

In epithelia and endothelia, AJ is localized below TJ in the basolateral region of two adjacent cells. It is a discrete structure physically segregated from TJ and is primarily responsible for cell–cell adhesion by connecting to a dense actin cytoskeleton that create a plaque-like ultrastructure known as zonula adherens (Hartsock and Nelson, 2008; Miyoshi and Takai, 2008). In the testis, however, AJ is distinctly different from those found in other epithelia/endothelia, instead a testis-specific ultrastructure known as ES is found. There are two ESs in the seminiferous epithelium dependent on its location. The ES that is found near the basement membrane between adjacent Sertoli cells, and is localized at the BTB is the basal ES, it coexists with TJ and GJ, and is responsible for Sertoli cell–cell adhesion (Cheng

and Mruk, 2010b). The ES that is localized to the apical compartment and is the only anchoring device between Sertoli cells and spermatids (steps 8–19 in the rat testis) is the apical ES. ES is associated with an extensive actin filaments arranged in hexagonal bundles with unipolar orientation that lie perpendicular to the Sertoli cell plasma membrane (Mruk et al., 2008; Yan et al., 2007). Interestingly, these actin filaments are noncontractile in nature, thus they are not likely to be involved in germ cell movement as developing germ cells are immobile cells per se, lacking all the cell movement apparatus (e.g. lamellipodia) and Sertoli cells inside the seminiferous epithelium are also not actively motile cells per se (Mruk et al., 2008; Yan et al., 2007). As the actin filament bundles at the ES are restricted *only* to the Sertoli cell, but not in elongating/elongated spermatids, the ultrastructural features of the apical ES and basal ES are essentially identical except that actin filament bundles are found on both sides of Sertoli cells at the basal ES, but restricted only to the Sertoli cell at the apical ES (Cheng and Mruk, 2010b). Interestingly, the protein composition in both apical and basal ESs is quite different (Cheng and Mruk, 2010b). For instance, JAM-C, nectin-3, β1-integrin, laminin-α3,-β3,-γ3 are restricted to the apical ES, and JAM-A and -B are limited to the basal ES, whereas other proteins, such as CAR, are found in both apical and basal ES (Cheng and Mruk, 2010b). At the apical ES, other than AJ proteins that are usually found in epithelia/endothelia (e.g. N-cadherin, β-catenin, nectin-2), TJ proteins, GJ proteins, and focal adhesion complex (FAC, which is an anchoring junction at the cell–matrix interface) proteins are also found, making this a hybrid junction (Mruk et al., 2008; Wong et al., 2008; Yan et al., 2007).

2.2.1. Basal ES

The basal ES is restricted to adjacent Sertoli cells near the basement membrane at the site of the BTB, which is typified by the bundles of actin filaments sandwiched in-between cisternae of endoplasmic reticulum and the two opposing plasma membranes of Sertoli cells (Cheng and Mruk, 2010b). While the ultrastructural features of basal ES are indifferent from the apical ES, their constituent proteins are quite different as the basal ES shares some similarity with conventional AJ. For instance, constituent adhesion molecules at the basal ES are members of the cadherins and nectins family.

2.2.1.1. Cadherins

Being one of the major constituent proteins of AJs, the importance of cadherins is well demonstrated by the embryonic lethality of mice lacking

classical cadherins, such as E-cadherin and N-cadherin (Radice et al., 1997). In rodent testis, the above two classical cadherins are found at the basal ES (Mruk et al., 2008; Yan et al., 2007). They are single span membrane protein having a divergent extracellular domain containing five repeats called ectodomain modules (ECs) and a conserved cytoplasmic tail (Harris and Tepass, 2010; Yonemura, 2011). Binding of Ca^{2+} ions is necessary for correct protein confirmation of the ECs, which participate in forming homotypic *cis*-dimers of cadherins on the same side of two neighboring cells. Two *cis*-dimers of cadherins from each adjacent cells then form homotypic *trans*oligomers that create an AJ (Harris and Tepass, 2010; Yonemura, 2011). Although the binding between cadherin extracellular domains is weak, cell–cell adhesion is strengthened via lateral clustering of cadherins, which is a process mediated by nectins (Sakisaka et al., 2007; Takai et al., 2008). Cadherin clustering also required binding of p120-catenin and β-catenin to cadherin juxtamembrane region and cytoplasmic tail, respectively. p120-catenin is essential for the retention of cadherins at the plasma membrane. Studies using siRNA to knockdown p120-catenin or by overexpressing exogenous cadherins have shown that p-120 catenin–cadherin association is able to stabilize the cadherins by preventing cadherins at the cell surface from being internalized and degraded (Davis et al., 2003; Iyer et al., 2004; Maeda et al., 2006). On the other hand, β-catenin–cadherin association promotes cadherin clustering by connecting cadherins to actin cytoskeleton through the adaptor α-catenin, which can bind β-catenin and also actin filaments (Harris and Tepass, 2010; Yonemura, 2011). Studies have shown that during formation of AJs which is initiated by nectins, clustering of cadherins is aided by remodeling of actin cytoskeleton via actin regulating proteins such as the Arp2/3 complex which induces branched actin polymerization for capturing clusters of cadherins (Kametani and Takeichi, 2007; Le Clainche et al., 2007; Sato et al., 2006). However, a disruption of cortical actin filaments can lead to dissolution of cadherins at the cell–cell interface (Quinlan and Hyatt, 1999), illustrating the importance of actin filament network in recruiting cadherin-based AJs to cell–cell interface. It was long believed that AJs were maintained through the association of cadherin–β-catenin–α-catenin complex to actin filaments. However, it is now known that α-catenin cannot simultaneously bind to β-catenin and actin, implying a cadherin-β–catenin–α-catenin–actin association does not exist (Drees et al., 2005). Instead, α-catenin exists as monomers and dimers, which bind to β-catenin and actin, respectively. Clustering of cadherin-β–catenin–α-catenin complex during AJ formation induces a localized

concentrated pool of α-catenin that favors its dimerization. Thus, α-catenin dissociates from β-catenin and forms dimers, which in turn associate with actin filaments. Association of α-catenin to actin filament inhibits the activity of the Arp2/3 complex and hence, reorganizing F-actin network from a "branched" to a "bundled" conformation (Drees et al., 2005), thereby stabilizing cell–cell adhesions with bundles of cortical actin filaments. In this context, it is of interest to note that while AJs may connect to the actin cytoskeleton via the nectin–afadin complex, the strong adhesion provided by AJs in an epithelium is difficult to achieve without the cadherin–β-catenin–α-catenin–actin association (Harris and Tepass, 2010). Moreover, when the actin-binding domain of α-catenin is deleted, the directional movement of cadherin–α-catenin fusion proteins to the apical junctional complex is abolished, illustrating binding of α-catenin to actin filaments is essential for actin cytoskeleton-mediated lateral flow of cadherins (Kametani and Takeichi, 2007). It seems that there are missing links regarding how α-catenin connects the cadherin–β-catenin complex to actin cytoskeleton, and additional research is needed in this area.

2.2.1.2. Nectins

Nectins are a family of immunoglobulin-like cell adhesion molecules with four members known to date, namely nectin-1 to -4. In general, each nectin has an extracellular domain which contains three Ig-like loops, a transmembrane region and a cytoplasmic tail (Sakisaka et al., 2007; Takai et al., 2008). Each nectin member first forms homotypic *cis*-dimers, which in turn form homotypic or heterotypic *trans*-dimers in a Ca^{2+}-independent manner. Interestingly, the adhesive force between heterotypic *trans*-dimers is stronger than that between homotypic *trans*-dimers (Sakisaka et al., 2007; Takai et al., 2008). Nectins are connected to actin cytoskeleton via a cytoplasmic adaptor afadin (Sakisaka et al., 2007; Takai et al., 2008). Besides binding to nectins via PDZ domain and actin filaments via its C-terminal tail, afadin indeed has multiple domains, enabling it to associate with different proteins, such as c-Src, Rap1 (a small G protein), ZO-1, α-catenin (Sakisaka et al., 2007; Takai et al., 2008). This thus mediates signal transduction and provides cross talk between cadherin- and nectin-based junctions. Studies have demonstrated that by coupling with actin reorganization, nectins are responsible for initiating AJ formation and for recruiting cadherins to complete the process. As epithelial cells initiate cell–cell contact, *trans*-interacting nectins from adjacent cells were found to activate Cdc42 (a small GTPase of the Rho-subfamily), Rac (also a signaling GTPase) via

c-Src in an afadin-independent manner (Fukuyama et al., 2005; Kawakatsu et al., 2005, 2002). Activated Cdc42 and Rac, in turn, trigger reorganization of actin cytoskeleton through the actin-binding protein IQGAP1, which induce branched actin polymerization via the Arp2/3 complex (Le Clainche et al., 2007; Sato et al., 2006) to recruit cadherins to the site. It is noted that at this step, the recruited cadherins are non-*trans*-interacting since they have yet to associate with cadherins from neighboring cells. Clustering of these non-*trans*-interacting cadherins is then assisted by afadin-associated *trans*-interacting nectins. This is achieved by activation of Rap1 by *trans*-interacting nectins, activated Rap1 then associates with afadin to form a complex, which in turn binds to p120-catenin to retain cadherins at plasma membrane (Hoshino et al., 2005; Sato et al., 2006). Hence, localized clustering of cadherins takes place which favors the *trans*-interaction of cadherins to establish AJs.

Nectin-2 is expressed in rodent Sertoli cells (Bouchard et al., 2000; Ozaki-Kuroda et al., 2002). Mice lacking nectin-2 are infertile illustrating nectin-2 is indispensable for spermatogenesis (Bouchard et al., 2000; Ozaki-Kuroda et al., 2002). Although studies of mice lacking nectin-2 were focused on apical ES (Kawakatsu et al., 2002) or spermatids (Bouchard et al., 2000), it was noted that the actin filament bundles at the apical ES in these mice were absent, suggesting that their BTB might have been disrupted due to a disorganized actin cytoskeleton.

2.2.1.3. Interplay between AJs and TJs Via Adaptor Proteins

As noted above, cell adhesion molecules cross talk with each other via their peripheral adaptors to maintain epithelial homeostasis. For instance, AJs are crucial for TJ assembly, and ZO-1 is a crucial player in this process (Hartsock and Nelson, 2008; Sakisaka et al., 2007). Studies have shown that nectin–afadin complex is able to recruit ZO-1, which was then used to recruit JAMs, claudins and occludin to the apical junctional complex to form TJs (Ooshio et al., 2010; Yokoyama et al., 2001). The necessity of *trans*-interacting nectins in the establishment of TJs was demonstrated when such interaction was blocked via the use of a chimeric protein that bound to the extracellular region of nectins, the recruitment of JAMs (Fukuhara et al., 2002a), claudins and occludin (Fukuhara et al., 2002b) for TJ assembly was impaired. Moreover, the importance of *trans*-interacting nectin–afadin association in initiating TJ assembly was shown by expressing nectins with a truncated C-terminus, rendering nectins incapable of binding to afadin, leading to an impairment to recruit ZO-1 to establish TJs (Yokoyama et al., 2001). Furthermore,

interaction between afadin and ZO-1 is important for TJ assembly since a knockdown of either afadin or ZO-1, or over-expression of a truncated form of afadin that failed to bind to ZO-1 after the knockdown of endogenous afadin, impeded TJ formation (Ooshio et al., 2010). Besides playing a crucial role in TJ assembly, AJs are also essential for TJ maintenance, as a disruption of AJs often leads to TJ disassembly. For instance, when E-cadherin-mediated cell–cell adhesion was inhibited by treatment of an anti-E-cadherin antibody (Man et al., 2000), or when E-cadherin was downregulated after depletion of cellular polyamines (Guo et al., 2003), a disruption of the TJ-permeability barrier was detected, illustrating a primary loss of AJ function leads to a secondary dysfunction of TJs. More important, cross talk between AJs and TJs is not unidirectional since AJ integrity is also dependent on the integrity of TJs. For instance, downregulation of occludin induced by transfecting PA4 (polyaxonal amacrine 4 cells of retina) epithelial cells with Raf-1, mislocalization of E-cadherin was observed, suggesting AJ disruption (Li and Mrsny, 2000). Collectively, these findings illustrate that while TJs and AJs are found in discrete locations in epithelia/endothelia, they are still functionally connected via their peripheral adaptor proteins. At the BTB, TJ and basal ES *coexist* in the same location, and such intimate relationship is especially important to elicit transient "opening" and "closing" of the barrier during the transit of preleptotene spermatocytes at stage VIII–IX of the epithelial cycle. It was noted that treatment of adult rats with adjudin at 50 mg/kg b.w. that was effective to induce germ cell loss from the epithelium except spermatogonia (Mok et al., 2012b; Yan and Cheng, 2005) did not impede the BTB integrity. During the process of adjudin-induced germ cell loss, the adaptor proteins α-catenin and ZO-1 at the basal ES and TJ, respectively, which were originally tightly associated ("engaged") for linking basal ES and TJ together to reinforce the BTB integrity, became dissociated ("disengaged"). Thus, a primary disruption of the apical ES at the Sertoli–spermatid interface that facilitates germ cell loss do not perturb the TJ-barrier function at the BTB since the adaptors that link basal ES (e.g. catenins) and TJ (e.g. ZO-1) together are "disengaged" during adjudin-induced germ cell loss (Yan and Cheng, 2005). This thus illustrates that a novel mechanism is in place in the testis to safeguard the BTB integrity in response to changes in environment, such as following exposure to a toxicant, or during the epithelial cycle of spermatogenesis, when spermatids are in transit across the seminiferous epithelium involving localized apical ES restructuring, so that the BTB integrity can be maintained via "disengagement" of basal ES and TJ proteins.

2.2.2. Apical ES

In rodents, the apical ES, once it appears, is the only anchoring device between Sertoli cells and elongating spermatids (step 8–19 in rats). Besides conferring adhesion and structural support to developing spermatids, the apical ES also confers spermatid polarity during spermiogenesis so that the heads of developing spermatids are pointing toward the basement membrane, thus, the maximal number of spermatids can be packed in the seminiferous epithelium of a tubule (Wong and Cheng, 2009). Although the actin filament bundles, the hallmark ultrastructure of the ES, are only visible on the Sertoli cell, not the spermatid, at the apical ES (Cheng and Mruk, 2010b; Mruk et al., 2008), but the stage-specific expression of cadherins (Johnson and Boekelheide, 2002; Lee et al., 2003), nectin-3 (Ozaki-Kuroda et al., 2002) and laminin-α3, -β3, and γ-3 chains (Koch et al., 1999; Siu and Cheng, 2004;Yan and Cheng, 2006) by the spermatids during the epithelial cycle suggest that spermatids also play a role in establishing the apical ES. Apical ES is the strongest anchoring devices between Sertoli cells and spermatids (steps 8–19), significantly stronger than DSs between Sertoli cells and spermatids (steps 1–7) (Wolski et al., 2005). This unusual adhesive force is contributed by a number of factors. For instance, nectin-3 is exclusively expressed by elongating/elongated spermatids in the testis and this enables the formation of heterotypic *trans*-interaction between nectin-3 from germ cells and nectin-2 from Sertoli cells to yield a strong cell–cell adhesion. Furthermore, the hybrid nature of the apical ES also supports its adhesive strength. Among the different junction proteins present at the apical ES, it is believed that the interaction between laminin-333 (composed of laminin α3, β3, γ3 chains) from elongating/elongated spermatids and the α6β1-integrin from Sertoli cells contribute significantly to its adhesive force (Palombi et al., 1992; Salanova et al., 1995;Yan and Cheng, 2006). Interestingly, besides performing the anchoring function at apical ES, the laminin-333–α6β1-integrin protein complex also participates in regulating BTB integrity at the apical ES–BTB–hemidesmosome axis (Fig. 6.2). It was proposed that during spermiation, laminin chains at the apical ES was cleaved by matrix metalloproteinases, such as MMP-2, which was highly expressed at the apical ES at stage VIII of the epithelial cycle (Siu and Cheng, 2004), to facilitate the release of mature spermatids at spermiation (Yan et al., 2008a). Some of these fragments of laminin chains, which were shown to regulate cell-adhesion function in other epithelia (Yan et al., 2008b) were shown to perturb the Sertoli cell TJ-permeability barrier function (Yan et al., 2008a). This functional axis between the apical ES and the BTB was confirmed by

adding purified recombinant laminin fragments into Sertoli cell cultures with an established TJ barrier, which was shown to disrupt the TJ barrier in vitro via down-regulation of integral membrane proteins occludin and JAM-A at the BTB, and similar observations were obtained by overexpressing these laminin fragments in Sertoli cells (Yan et al., 2008a). Surprisingly, laminin fragments were also found to reduce the level of β1-integrin at the hemidesmosome (an intermediate filament based cell–matrix anchoring junction present at the Sertoli cell–basement membrane interface) (Yan et al., 2008a). A knockdown of β1-integrin at the hemidesmosome in Sertoli cell epithelium in vitro also led to a disruption of the TJ barrier via redistribution of occludin and N-cadherin, with these proteins moved from the cell–cell interface into the cell cytosol (Yan et al., 2008a), illustrating there is a functional link between the hemidesmosome and the BTB. These findings thus illustrate that while spermiation and BTB restructuring that take place at the opposite ends of the epithelium at stage VIII of the epithelial cycle, they are functionally connected via the apical ES–BTB–hemidesmosome axis. The presence of this axis was recently confirmed by using a Sertoli cell injury model using phthalates, in which phthalate-induced apical ES disruption that led to spermatid lose and accompanied by a reducing level of laminins also induced a MMP-mediated BTB disruption (Yao et al., 2009, 2010).

2.3. Gap Junctions

The building blocks of GJs are integral membrane proteins known as connexins (Cx) such as Cx26, 33, 43. Six connexins form a hemichannel called *connexon*, and a connexon from one cell that docks with another connexon of an apposing or adjacent cell forms a functional GJ (Enders, 1993; Li et al., in press; Pointis et al., 2010). The primary function of GJs is to act as communicating channels between neighboring cells for mediating cell–cell communication for signal transduction (Bosco et al., 2011; Giepmans, 2004). In general, these channels allow diffusional exchange of ions and small molecules that are <1 kD in size, however, GJs assembled by different connexins indeed have variations among their pore size (Bosco et al., 2011; Giepmans, 2004). More than 20 connexins have been identified in rodent and human genomes. GJ can be composed of homotypic or heterotypic connexons, as such, a variety of GJs can be produced. Additionally, control of passage of molecules across GJs can be further modulated in a connexin-specific manner (Bosco et al., 2011; Giepmans, 2004). GJs can also interact with AJs and TJs through the shared adaptor ZO-1. Thus, ZO-1 also link

GJs to actin cytoskeleton, which is important for proper localization of GJs (Giepmans and Moolenaar, 1998; Laing et al., 2001; Toyofuku et al., 1998). Besides mediating signaling between neighboring cells, GJs are also involved in modulating the function of AJs and TJs (Derangeon et al., 2009; Kojima et al., 2007) including TJ-barrier function at the BTB (Li et al., 2009). Studies have shown that in cultured Sertoli cells, a transient induction of Cx33 coincides with a surge in the expression of N-cadherin (Chung et al., 1999), and blocking the *trans*-interaction of connexons with synthetic peptides leads to mislocalization of N-cadherin (Lee et al., 2006), illustrating the involvement of GJs in the assembly and maintenance of AJs in the testis. Furthermore, the requirement of GJs in inducing TJ assembly and its maintenance was revealed in studies via overexpression of exogenous Cx32 in hepatocytes isolated from Cx32-deficient mice that led to an induction of TJs in these cells (Kojima et al., 2002). Furthermore, a disruption of GJ-communication in Caco-2 cells (human colonic epithelial cell line) resulted in TJ-barrier disruption (Morita et al., 2004). These studies illustrate GJ proteins themselves and/or GJ-mediated cell–cell communication is essential to the assembly and/or maintenance of AJs and TJs. Thus, GJs are expected to be crucial for BTB maintenance during spermatogenesis. In fact, spermatogenesis was disrupted in mice with Sertoli cell-specific deletion of Cx43 (Brehm et al., 2007; Carette et al., 2010). In these Cx43 SC only KO mice, spermatogenesis was arrested in which spermatogonia failed to differentiate beyond type A (Carette et al., 2010). Furthermore, a knockdown of Cx43 in cultured Sertoli cells with an established functional TJ-permeability barrier by RNAi perturbed the "resealing" of a disrupted TJ barrier induced by either Ca^{2+} depletion or treatment with bisphenol A (Li et al., 2010). Such a loss of the ability of the Sertoli cell to "reseal" the disrupted TJ barrier following Cx43 knockdown was shown to be mediated, at least in part, by changes in the localization of AJ and TJ proteins at the BTB, rendering their BTB proteins incapable of redistributing to their proper sites to "reseal" the disrupted BTB (Li et al., 2010). Moreover, in cultured Sertoli cells, the simultaneous knockdown of both Cx43 and plakophilin-2 (PKP-2 a desmosomal adaptor protein) was found to induce mislocalization of TJ proteins occludin and ZO-1, as well as an increase in endocytosis of N-cadherin, thereby destabilizing the TJ barrier (Li et al., 2009). Thus, these findings are consistent with studies in other epithelia that GJs are required for proper functioning of basal ES and TJs at the BTB in the rat testis, possibly mediated by transmitting signals among different junction types to coordinate their functions to maintain the BTB homeostasis during the epithelial cycle of spermatogenesis.

3. MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

3.1. Introduction

The discovery of TOR, a Ser/Thr protein kinase, in yeasts was aided by using an antibiotic called rapamycin, which was found to specifically inhibit the activity of TOR and was thus designated "target of rapamycin (TOR)." Subsequent studies have identified its homolog in mammalian cells designated mammalian target of rapamycin (mTOR) (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994). Much attention was drawn to mTOR for its essential role in cell growth and proliferation as mTOR is the key regulator for sensing and integrating diverse environmental clues including growth factors, mitogens and nutrients so that appropriate cellular responses can occur in response to these changes (Laplante and Sabatini, 2012). Subsequent studies have shown that mTOR, besides protein synthesis that affects cell growth and proliferation, is virtually involved in almost all aspects of cellular function such as actin cytoskeleton reorganization, cell survival, and autophagy (Appenzeller-Herzog and Hall, 2012; Chi, 2012; Laplante and Sabatini, 2012; Nair and Ren, 2012), as well as pathogenesis such as carcinogenesis (Ekman et al., 2012; Fasolo and Sessa, 2012; Lieberthal and Levine, 2012; Posadas and Figlin, 2012; Sheppard et al., 2012). Dysregulation of mTOR signaling is observed in different pathological conditions, such as diabetes, cancer and obesity (Weichhart, 2012; Zoncu et al., 2011). mTOR belongs to PIKK (PI3K-related kinase) superfamily as its C-terminus shares strong homology to the catalytic domain of PI3K. However, instead of being a lipid kinase, mTOR is a Ser/Thr protein kinase. In order to execute its cellular functions, mTOR forms one of the two complexes, namely mTORC1 and mTORC2, by associating with different binding partners (Dazert and Hall, 2011; Laplante and Sabatini, 2012). mTORC1 is composed of mTOR, regulatory associated protein of mTOR (raptor), PRAS40, mLST8 and deptor. mTORC1 is responsible for the well-known roles of mTOR that regulates cell growth and proliferation by modulating protein synthesis. Moreover, mTORC1 is sensitive to rapamycin, which acts as an allosteric inhibitor for mTORC1 by associating with FKBP12 to form a complex. This complex binds to mTOR in a short stretch of sequence near its C-terminus known as the FKBP12–rapamycin-binding domain, causing dissociation of raptor from mTORC1 (Senqupta et al., 2010; Zhou and Huang, 2010). And for another mTOR complex, the mTORC2 was first described as rapamycin insensitive as

FKBP12–rapamycin complex does not bind to mTORC2 (Oh and Jacinto, 2011; Zhou and Huang, 2010). The key binding partner of mTORC2 is rictor (rapamycin-insensitive companion of mTOR). Unlike mTORC1, mTORC2 regulates actin cytoskeleton and cell survival. Besides rictor, other subunits of mTORC2 include Sin1, mLST8, deptor, Hsp70 and protor-1/2. Interestingly, subsequent studies have shown that while mTORC2 is insensitive to rapamycin, but this is limited to short-term exposure since prolonged rapamycin challenge at up to 24 h leads to the dissociation of rictor from mTOR, disabling the mTORC2 signaling (Sarbassov et al., 2006). Although FKBP12–rapamycin complex does not bind to mTORC2, it was proposed that after long-term treatment, the availability of mTOR decreased as newly synthesized mTOR was occupied by FKBP12–rapamycin complex, preventing the formation of mTORC2. Different binding partners among mTORC1 and mTORC2 allow these kinases responding to different stimulating signals so that they can phosphorylate unique sets of substrates to induce distinctive physiological responses.

3.2. Mammalian Target of Rapamycin Complex 1 (mTORC1)

mTORC1 is composed of mTOR, raptor, proline-rich Akt/PKB substrate 40 kDa (PRAS40), mTOR associated protein LST8 homolog (mLST8) and DEP domain-containing mTOR-interacting protein (deptor) (Fig. 6.3). Among them, raptor is the key binding partner which acts as a critical scaffolding protein that controls mTORC1 assembly and the selection of substrates (Kim et al., 2002; Nojima et al., 2003; Schalm et al., 2003). In the absence of nutrients, raptor associates with mTOR stably to repress mTORC1 catalytic activity while under nutrient-rich conditions, the binding of raptor to mTOR is unstable but this unstable mTOR–raptor association is necessary for mTORC1 to carry out its kinase activity (Kim et al., 2002). Raptor can be phosphorylated at multiple sites for either up- or down-regulating mTORC1 activity (Zhou and Huang, 2010). For instance, under energy stress conditions, AMP-activated protein kinase (AMPK) phosphorylates raptor on S722 and S792 to induce binding of 14-3-3 protein to mTORC1 to elicit its inhibition, leading to cell cycle arrest (Gwinn et al., 2008). Activation of mTORC1 by mitogens, however, is mediated via phosphorylation of raptor on S719, S721 and S722 by p90 ribosomal S6 kinases (RSKs) (Carriere et al., 2008). Deptor (an inhibitor of mTOR) and mLST8 are common subunits among mTORC1 and mTORC2. Deptor binds to both mTOR complexes and functions as a negative regulator (Peterson et al., 2009). For mLST8, it is required for mTORC2 to maintain its

activity (Guertin et al., 2006). However, the necessity for mLST8 in activating mTORC1 signaling remains unclear. The binding of mLST8 to mTORC1 was shown to stimulate mTORC1's kinase activity toward S6K1 and 4E-BP1 (Kim et al., 2003). However, in mLST8-deficient fibroblasts, the association between mTOR and raptor, as well as the phosphorylation of substrates of mTORC1 are not impaired, indicating mLST8 has limited function for mTORC1 in fibroblasts (Guertin et al., 2006). Thus, it is of interest to determine whether there are mLST8-like protein(s) to rescue the function of mTORC1 in mLST8-deficient fibroblasts (Guertin et al., 2006). PRAS40 is another negative regulator of mTORC1 (Oshiro et al., 2007; Wang et al., 2007). PRAS40 inhibits mTORC1 activity by binding to mTORC1 via raptor, and phosphorylation of PRAS40 by PKB leads to its detachment from mTORC1, activating the complex (Wang et al., 2008). When mTORC1 is activated by appropriate signals, mTORC1 induces cell growth and proliferation via up-regulation of protein synthesis by phosphorylating S6 protein kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Dazert and Hall, 2011; Laplante and Sabatini, 2012).

3.2.1. Upstream Signaling Molecules of mTORC1

As noted above, the activity of mTORC1 is modulated by stimuli such as growth factors, mitogens, amino acids and energy status (Fig. 6.3). For the growth factors that trigger mTORC1 signaling, insulin is among the best studied (Magnuson et al., 2012; Zoncu et al., 2011). Upon binding of insulin or insulin-like growth factor (IGF) to its receptors, autophosphorylation of these receptors takes place, which then phosphorylates the insulin receptor substrates (IRS). Activated IRS in turn phosphorylates PI3K, which catalyzes the conversion of phosphatidylinositol (4, 5)-bisphosphate (PIP_2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP_3). This conversion can be reversed by phosphatases and tensin homolog on chromosome 10 (PTEN), which is an important negative regulator of mTORC1 pathway by converting PIP_3 to PIP_2, thus dysregulation of PTEN is detected in numerous kinds of cancer (Song et al., 2012). PIP_3 recruits 3-phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate PKB on T308 and for full activation, PKB is then phosphorylated by another kinase on S473 (Alessi et al., 1997; Andjelkovic et al., 1997) (Fig. 6.3). Activated PKB phosphorylates and inhibits tuberous sclerosis complex 2 (TSC2), which associates with TSC1 to form a complex that inhibits mTORC1 (Manning et al., 2002). As GTP-bound Ras-homolog enrich in brain (Rheb) is required for the activation of mTORC1, the inhibitory effect of TSC1/2 complex is mediated via its GTPase activity that acts on Rheb to maintain Rheb in a GDP-bound

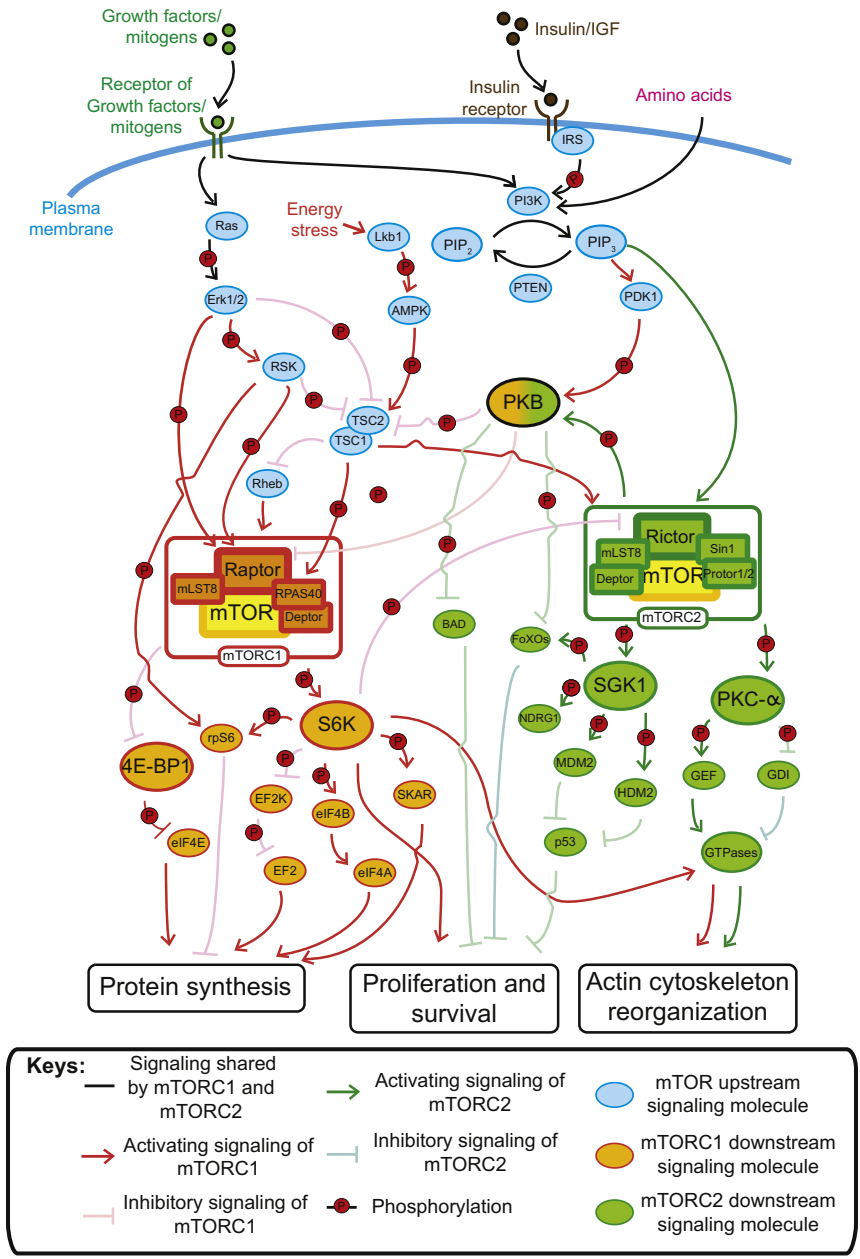

Figure 6.3 ***The likely mTOR signaling pathways involving mTORC1 and mTORC2 and the corresponding interacting/regulatory proteins that regulate different cellular events including BTB function in the testis via the effects on F-actin organization.*** By assembling with different subunits, two mTOR complexes can be formed, namely, mTORC1 and

status. After the phosphorylation of TSC2, TSC1/2 complex is inhibited and hence, Rheb-GTP is accumulated for the activation of mTORC1.

In addition to TSC1/2 complex, PKB also promotes mTORC1 signaling by phosphorylating PRAS40. As such, PRAS40 is dissociated with raptor and hence, its inhibitory effect is removed (Wang et al., 2008). Moreover, besides the above PKB-mediated pathways, binding of growth factors or mitogens to their receptors can also activate mTORC1 via the Ras-Raf-MEK-ERK signaling. Upon the above activation, the small GTPase Ras eventually leads to phosphorylation of ERK1 and ERK2, which in turn inhibits the TSC1/2 complex by directly phosphorylating TSC2 or via activation of RSK1 that also phosphorylates and inhibits TSC2 (Ma et al., 2005; Roux et al., 2004). Furthermore, ERK1/2 and RSK1 also phosphorylate raptor to promote mTORC1 functions (Carriere et al., 2008, 2011) (Fig. 6.3). While mTORC1 signaling has to be "on" to upregulate protein synthesis in response to growth factors and mitogens, there are conditions that mTORC1 pathway has to be "off," for example, when cells are in energy stress. When cellular ATP decreases, the rise of AMP/ATP ratio activates Lkb1 (liver kinase B1, also known as Ser/Thr kinase 11, STK11) to phosphorylate AMPK (Shackelford and Shaw, 2009), which in turn phosphorylates raptor, inhibiting mTORC1 as mentioned above (Gwinn et al., 2008). Besides, AMPK phosphorylates and activates TSC2, thus mTORC1 is suppressed by the TSC1/2 activity which catalyzes the conversion of Rheb-GTP to Rheb-GDP (Inoki et al., 2003).

3.2.2. Downstream Signaling Molecules of mTORC1

3.2.2.1. S6 Protein Kinases

Upon activation, mTORC1 up-regulates protein synthesis mainly through its two substrates S6K and 4E-BP1. In mammals, S6K is a family of protein

mTORC2. Besides mTORC1 that is specifically regulated by the energy status of a cell, both mTOR complexes are activated by growth factors (e.g. insulin), mitogens and amino acids. Upon activation, except that upregulation of protein synthesis for cell growth is specifically mediated by mTORC1, the control of cell proliferation and survival as well as actin cytoskeleton organization is modulated by both complexes, despite the fact that they have their unique substrates and downstream signaling molecules. Moreover, mTORC1 and mTORC2 share several upstream signaling molecules. For example, PIP_3 can activate both complexes while TSC1/2 complex *inhibits* mTORC1 but *activates* mTORC2. Furthermore, the signaling pathways of the two mTOR complexes are interconnected in which S6K1, the substrate of mTORC1, is able to phosphorylate rictor and thus inhibits mTORC2. As such, phosphorylation of PKB, which is the substrate of mTORC2, can be reduced. Since PKB phosphorylation is required for activating mTORC1, this leads to suppression of mTORC1 signaling and therefore, a negative feedback loop is established. For color version of this figure, the reader is referred to the online version of this book.

kinases that has two members, namely S6K1 and S6K2, which are encoded by two different genes and share high homology (Lee-Fruman et al., 1999). The necessity of S6K in regulating cell and body size is well illustrated in genetic models deficient in either S6K1 or S6K2, or both. The loss of function of S6K in *Drosophila* led to a serve decline in body size due to reduction in cell size rather than cell number (Montagne et al., 1999). In mice lacking S6K1, body size was significantly smaller *versus* the wild type at birth and during postnatal growth, attributing by the reduced size in all organs (Shima et al., 1998). The reduction in size of organs was probably caused by decreased cell size, such as pancreatic β-cells (Pende et al., 2000) and myoblasts (Ohanna et al., 2005). In contrast, in mice lacking S6K2, body size was insignificantly different from the wild type at birth and during postnatal development, suggesting S6K2 is not required for regulating cell size in rodents. Furthermore, in mice deficient in both S6K1 and S6K2, body size of the surviving animals during embryonic and postnatal growth was not further reduced compared to that of S6K1-deficient mice (Pende et al., 2004) and the size of myoblasts from S6K1- and S6K2-deficient mice was similar to that of mice lacking S6K1 (Pende et al., 2004). These findings thus suggest that control of cell size seems to be mostly regulated by S6K1 in rodents. Moreover, it has been reported that mice lacking both forms of S6K are prone to suffer perinatal death, unlike mice lacking either form of S6K, which were viable and fertile (Pende et al., 2004). This indicates that although S6K2 may not contribute as much as S6K1 in regulating cell size, these two isoforms do have overlapping roles and therefore, loss of one isoform can be superseded, at least in part, by the other.

Ribosomal protein S6 (rpS6) was the first identified substrate of S6K1 for modulating protein synthesis (Gressner and Wool, 1974). Subsequent studies have identified other substrates of S6K1, which include elongation factor 2 (EF2) kinase, eukaryotic initiation factor 4B (eIF4B), programmed cell death 4 (PDCD4) and S6K Aly/REF-like substrate (SKAR) that promote protein synthesis via up-regulating translational activity. It is known that S6K1 phosphorylates and inactivates EF2 kinase (EF2K), leading to dephosphorylation and activation of EF2, which in turn promotes translation elongation (Wang et al., 2001). S6K1 also phosphorylates eIF4B on S422, resulting in enhanced translation initiation by stimulating the RNA helicase eIF4A to unwind mRNA for translation (Raught et al., 2004). The above process is further enhanced by phosphorylating the eIF4A inhibitor, PDCD4 (note: each PDCD4 molecule can bind two molecules of eIF4A) by S6K1 on S67 as such phosphorylation promotes PDCD4 degradation (Dorrello et al., 2006; Shahbazian et al., 2006). Furthermore, studies revealed that S6K1 also promoted protein translation by

phosphorylating SKAR on S383 and S385 (Richardson et al., 2004). It is of interest to point out that SKAR was shown to be specifically phosphorylated by S6K1, but not S6K2, in regulating cell size (Richardson et al., 2004).

Besides regulating cell growth, S6K is also involved in stimulating cell proliferation. Rapamycin treatment has been shown to arrest cell cycle in mammalian lymphocytes at G_1 phase; however, rapamycin treatment only delays cell cycle progression in other mammalian cell types (Abraham and Wiederrecht, 1996). This indicates the significance of mTORC1 signaling in cell cycle progression and S6K is one of the mediators since G_1 phase progression was shown to be accelerated by overexpression of constitutively active S6K1 (Fingar et al., 2004). On the other hand, the importance of S6K2 in cell proliferation is illustrated in study demonstrating S6K2 was responsible for the interleukin-3 (IL-3)-driven cell proliferation since S6K2 was activated in lymphocytes and primary mouse bone marrow-derived mast cells upon IL-3 induced proliferation; and cell cycle progression was accelerated by overexpression of constitutively active S6K2 in lymphocytes (Cruz et al., 2005). Furthermore, the association of heterogeneous ribonucleoprotein (hnRNP) F with mTOR and S6K2, but not S6K1, is essential for driving cell proliferation (Goh et al., 2010). Taking collectively, both S6K1 and S6K2 are involved in mTORC1-mediated cell cycle progression. Interestingly, S6K1 is predominantly found in the cell cytosol versus S6K2 in the cell nucleus (Lee-Fruman et al., 1999).

3.2.2.2. Ribosomal Protein S6 (rpS6)

rpS6 was the first S6K substrate identified, and was thought to be its effector to upregulated protein synthesis (Magnuson et al., 2012). rpS6 is one of the ribosomal proteins of the 40S subunit of eukaryotic ribosomes (Wool, 1996). Much attention was given to rpS6 in the past since it was shown to undergo inducible phosphorylation upon a wide range of stimuli that upregulated protein synthesis (Gressner and Wool, 1974; Thomas et al., 1982; Wettenhall and Howlett, 1979; Wool, 1979). rpS6 can be phosphorylated in five residues located at the C-terminus: S235, S236, S240, S244 and S247 (Bandi et al., 1993; Krieq et al., 1988). It was suggested that phosphorylation progressed in an orderly manner that S236 is the primary phosphorylation site (Flotow and Thomas, 1992; Wettenhall et al., 1992). Full phosphorylation of rpS6 requires the presence of both S6K isoforms with S6K2 being the predominant kinase. However, studies reported in cells lacking both S6K or after rapamycin treatment wherein S6K activation was completely abolished, yet rpS6 was still being phosphorylated on S235 and S236. This thus illustrates

S6K is not the only kinase for rpS6 (Pende et al., 2004). Indeed, rpS6 can be phosphorylated by RSK (p90 ribosomal S6 kinase), via the Ras-Raf-MEK-ERK signaling (Roux et al., 2007) (Fig. 6.3). Being the substrate of both S6K and RSK, which are kinases that are known to upregulate protein synthesis, it was once believed that rpS6 promoted protein translation. It is because upon stimulation of cells by growth factors, mitogens and/or nutrients, rpS6 phosphorylation was positively correlated to translational activation of a class of mRNAs having characteristic 5′ terminal oligopyrimidine (TOP) tract, as both events took place simultaneously. These mRNAs, known as TOP mRNAs, are responsible for encoding numerous translational apparatus. Hence, based on the fact that rpS6 is a subunit of ribosome that undergoes phosphorylation during protein synthesis upregulation, rpS6 was thought to be responsible for stimulating the translation of TOP mRNAs (Meyuhas, 2000). Furthermore, translational activation of TOP mRNAs upon stimulation by mitogens was abolished by rapamycin treatment in some cell lines seemingly reinforced the above hypothesis (Hornstein et al., 2001). This concept, however, has been challenged by subsequent studies. First, in several cell lines, only a minor or no suppression of TOP mRNAs translation was found after rapamycin treatment, regardless of a complete activation blockage of S6K or its substrate rpS6 by rapamycin (Tang et al., 2001). Moreover, in amino acid starved cells, neither phosphorylation of rpS6 nor activation of S6K1 was sufficient to stimulate the translation of TOP mRNAs, whereas overexpression of dominant negative S6K1 which inhibited the activity of S6K1 and rpS6 phosphorylation failed to cause translational repression of TOP mRNAs in amino acid refed cells (Tang et al., 2001). Besides, even in dividing lymphoblastoids that S6K1 was active and rpS6 was phosphorylated, translation of TOP mRNAs was constitutively repressed (Stolovich et al., 2005). Furthermore, in some cell lines, the relief of translation repression of TOP mRNAs by LiCl was found to be independent of S6K and rpS6 (Stolovich et al., 2005). Collectively, these studies indicate that rpS6 phosphorylation is *not* indispensable for translational activation of TOP mRNAs and this possibility was validated by a study demonstrating that in mice expressing knockin nonphosphorylatable rpS6 ($rpS6^{p-/-}$), normal TOP mRNAs translation was detected (Ruvinsky et al., 2005). In short, it is increasingly clear that translational activation of TOP mRNAs is not mediated by rpS6 phosphorylation, and there is growing evidence to show that cell growth and even protein synthesis are not upregulated by phosphorylated rpS6, at least not in all mammalian cells. This notion is supported by studies using conditional rpS6 knockout mice or $rpS6^{p-/-}$ mice. It has been

reported that after fasting that caused loses in weight and protein content in liver, the liver mass and total protein content of both wild-type and rpS6 conditional knockout mice recovered to the same extent and at the same rate, clearly demonstrating rpS6 is dispensable for cell growth and protein synthesis (Volarevic et al., 2000). Furthermore, in liver, relative proportion of ribosomes associated with polysomes was similar between $rpS6^{p-/-}$ and wild-type mice (Ruvinsky et al., 2005). More importantly, in mouse embryonic fibroblasts (MEFs) that derived from $rpS6^{p-/-}$ mice, instead of protein synthesis retardation, a significant increase in rate of protein synthesis was observed (Ruvinsky et al., 2005). The studies using $rpS6^{p-/-}$ mice revealed that phosphorylation of rpS6 was not necessary for the efficient polysome recruitment for translation, and in fact protein synthesis was negatively regulated by phosphorylated rpS6. Therefore, it is now generally accepted that upon stimulations, such as by growth factors, mitogens and nutrients, that induce cell growth, mTORC1 upregulates protein synthesis via its substrates, S6K and 4E-BP1. The role of rpS6 is likely to fine tune the above process by playing a role as a negative regulator (Ruvinsky and Meyuhas, 2006). Similar to the kinase S6K, rpS6 may also be involved in the regulation of cell proliferation, such as proliferation of liver cells (Volarevic et al., 2000). Also, mouse embryonic fibroblasts derived from $rpS6^{p-/-}$ displayed an accelerated cell division, indicating rpS6 phosphorylation regulates cell proliferation negatively in these fibroblasts (Ruvinsky et al., 2005).

3.2.2.3. 4E-Binding Protein 1

Besides S6K, another well-characterized substrate of mTORC1 for mediating protein synthesis is 4E-BP1, which is a repressor of the translation initiation factor eIF4E (Pause et al., 1994). When mTORC1 signaling is not activated, eIF4E is sequestered by hypophosphorylated 4E-BP1. However, upon stimulation such as growth factors and mitogens, activated mTORC1 phosphorylates 4E-BP1 at six sites: T37, T46, T70, S65, S83 and S112, leading to dissociation of 4E-BP1 from eIF4E. eIF4E is thus free to bind to eIF4G, which is a scaffolding protein that recruits eIF4A and coordinates the binding of small ribosomal subunits to the mRNA. Association of eIF4E with eIF4G and eIF4A forms a complex called eIF4F which binds to the 5′-end of mRNA (Marcitrigiano et al., 1999) for the recruitment of 40S ribosome and eventually results in the formation of 48S translation preinitiation complex (Gingras et al., 1999).

Other than regulating cell growth and proliferation, mTORC1 signaling plays a wide variety of physiological roles including autophagy, aging,

memory and even actin reorganization (Weichhart, 2012; Zoncu et al., 2011). While mTORC1 and mTORC2 are two distinct signaling complexes having unique roles, they may work together in regulating many cellular events.

3.3. Mammalian Target of Rapamycin Complex 2 (mTORC2)

mTORC2 was discovered years after mTORC1, as such, less information is available for this signaling complex. Also, in contrast to the board downstream signaling molecules in the mTORC1 pathway, only a few substrates of mTORC2 have been identified, which include PKB, PKC-α and serum- and glucocorticoid-induced protein kinase 1 (SGK1) (Oh and Jacinto, 2011) (Fig. 6.3). mTORC2 signaling pathway is required for regulating cellular functions such as actin cytoskeleton organization and cell survival. Thus, malfunction of mTORC2 signaling is often found in different cancers with dysregulated actin organization and cell survival signals (Fang et al., 2012; Guo et al., 2012; Uesuqi et al., 2011). Besides the emerging necessities of mTORC2 for normal cell physiology, accumulating evidence has shown that these two mTOR complexes are interconnected, forming a complicated network of signaling molecules in mammalian cells in response to a wide range of stimuli.

Subunits of the mTORC2 include mTOR, rictor, Sin1 (stress-activated protein kinase (SAPK)-interacting protein 1), mLST8, deptor, Hsp70 and Protor-1/2. Among these, deptor and mLST8 are binding partners also found in mTORC1 and deptor serves as a negative regulator in both mTORC1 and mTORC2 (Peterson et al., 2009). While the function of mLST8 in mTORC1 is unclear, mLST8 is essential for the integrity of mTORC2 (Guertin et al., 2006). The importance of mLST8 to mTORC2 but not mTORC1 was revealed in a study in which raptor, rictor or mLST8 was deleted in mice. It was found that raptor-deficient mice died in early development; however, mice lacking mLST8 was able to survive until around embryonic day 10.5, similar to those lacking rictor, demonstrating the necessity of mLST8 to mTORC2 but not mTORC1 (Guertin et al., 2006). Also, upon knockout of mLST8, interaction between mTOR and raptor appeared to be normal and phosphorylation of S6K1 was not affected, whereas the association between mTOR and rictor, as well as the phosphorylation of PKB, were abolished (Guertin et al., 2006). Among the mTORC2 unique binding partners, rictor is the one that defines the function of mTORC2 by serving as a scaffolding protein for the assembly of the signaling complex (Powell et al., 2012). Mice lacking rictor led to a loss of PKB phosphorylation and embryonic lethality, demonstrating the significance of rictor for

the assembly of mTORC2 to regulate development (Guertin et al., 2006). Additionally, rictor has up to 37 phosphorylation sites with most of them are located at its C-terminus (Dibble et al., 2009). Activity of the mTORC2 can be regulated via these phosphorylation sites in response to different stimuli. For example, phosphorylation of T1135, which is sensitive to amino acid and growth factors, leads to reduced phosphorylation of PKB (Dibble et al., 2009; Julien et al., 2010). Another mTORC2 exclusive subunit that is essential for the stability of whole complex is Sin1 since a knockdown of Sin1 was found to disrupt the interaction between mTOR and rictor, reducing PKB phosphorylation (Yang et al., 2006). Additionally, Sin1 may be able to modulate the activity of mTORC2 through the phosphorylation status of rictor since following a knockdown of Sin1, phosphorylation of rictor was reduced (Yang et al., 2006). Moreover, it is of interest to know that five Sin1 isoforms are generated through alternative splicing, and at least three distinctive mTORC2 complexes can be formed by three of the Sin1 isoforms, each of which may have unique but yet-to-be identified functions (Frias et al., 2006). Hsp70 is another mTORC2-specific subunit that is required for the association between mTOR and rictor. It was shown that after knockdown of Hsp70, binding between mTOR and rictor, but not raptor, was disrupted (Martin et al., 2008). Besides, only the phosphorylation of PKB, but not S6K1, was reduced after knockdown of Hsp70, indicating Hsp70 is indispensable for mTORC2, but not mTORC1 function (Martin et al., 2008). For Protor-1/2, although it is a subunit of mTORC2, the loss of Protor-1, but not Protor-2, led to reduced phosphorylation of SGK1, but not PKB and PKC-α, revealing Protor-1 may be needed by mTORC2 to activate SGK1 efficiently (Pearce et al., 2011).

3.3.1. Upstream Signaling Molecules of mTORC2

mTORC2 was first identified as a protein Ser/Thr kinase that phosphorylated PKB specifically on S473, which was stimulated by growth factors after serum depletion (Sarbassov et al., 2005). Subsequent study has demonstrated that besides PKB, SGK1 is another substrate of mTORC2 (Garcia-Martinez and Alessi, 2008). Since growth factors are known to (i) activate SGK1 and mTORC2 and (ii) generate PIP_3 by PI3K, it was believed that PIP_3 was able to stimulate mTORC2 for activating SGK1 via an unknown mechanism(s). It is now known that PIP_3 can directly bind to and activate mTORC2 that leads to the phosphorylation of PKB and PKC-α by mTORC2 (Gan et al., 2011). Besides growth factors, mTORC2 can also be regulated by amino acids. After serum starvation, addition of Leu to HeLa cells is able to induce

mTORC2-dependent cell migration via actin-reorganization mediated by Rac (Hernandez-Negrete et al., 2007). Amino acids also induced phosphorylation of PKB on S473 by mTORC2 and such phosphorylation was perturbed by a knockdown of rictor (Tato et al., 2011). Other than growth factors and amino acids, mTORC1 and mTORC2 also share other common upstream stimuli/signaling molecules. It has been demonstrated that TSC1/2 complex, an inhibitor of mTORC1, can be physically associated with mTOR2. However, in contrast to its inhibitory effect on mTORC1, TSC1/2 complex binds and activates mTORC2 in a manner independent of its GTPase-activating protein activity (Huang et al., 2008) (Fig. 6.3). It was shown that a loss of TSC1/2 complex led to impaired phosphorylation of all the known downstream effectors of mTORC2 including PKB, PKC-α and SGK1, thus confirming the necessity of TSC1/2 complex in mTORC2 activation (Huang et al., 2009). In addition to TSC1/2 complex, PTEN, which is also a suppressor of mTORC1, seems to exert its effect upstream of mTORC2 (Fig. 6.3). Among the upstream signaling molecules of mTORC2, perhaps the most interesting is S6K1, which is also a substrate of mTORC1. Growth factors are known to induce phosphorylation of rictor at T1135, which is directly mediated by S6K1 in a rapamycin-sensitive manner (Dibble et al., 2009; Julien et al., 2010; Treins et al., 2010). Although such phosphorylation does not affect mTORC2 integrity, intrinsic kinase activity or cellular localization, it triggers the binding of rictor to 14-3-3 proteins, which is a family of regulatory molecules that modulate numerous cellular events including spermatogenesis in the testis (Hermeking, 2003; Sun et al., 2009). In addition, overexpression of rictor with nonphosphorylatable T1135 in wild-type or rictor-null cells led to an increase of PKB phosphorylation on S473 while the phosphorylation status of PKC-α and SGK1 remained unchanged, indicating phosphorylation of rictor by S6K1 may indeed negatively regulate the activation of PKB by mTORC2. The findings summarized herein illustrate mTORC1 and mTORC2 form a connected signaling network that the two signaling complexes interact with each other functionally (Fig. 6.3). For instance, as PKB is needed for stimulating mTORC1, the suppression of mTORC2 on PKB activation by the mTORC1 substrate S6K1 may act as a negative feedback system to prevent overactivation of mTORC1.

3.3.2. Downstream Signaling Molecules of mTORC2

PKB, PKC-α and SGK1 are the three known downstream effectors of mTORC2 and they are members of the AGC kinase (PKA, PKG, PKC) family (Fig. 6.3). AGC kinases have highly conserved primary sequence within their

kinase domains, and shared common structural features. For example, there is an activation loop in the catalytic domain of these molecules, and its phosphorylation leads to conformational changes which are essential to elicit the intrinsic catalytic activity of the enzyme (Parker and Parkinson, 2001; Pearce et al., 2010). Many AGC kinases also contain a hydrophobic motif located behind the kinase domain, and phosphorylation of this motif is required for stabilizing their active conformation. In addition, several AGC kinases have a turn motif (Parker and Parkinson, 2001; Pearce et al., 2010), which is an important phosphorylation site that promotes the integrity of the enzyme as well as maintaining its conformation for full kinase activity (Parker and Parkinson, 2001; Pearce et al., 2010).

3.3.2.1. Protein Kinase B

Among the substrates of mTORC2, PKB is the best characterized, which is known to be involved in regulating numerous cellular aspects including proliferation, survival, protein synthesis and metabolism. As mentioned previously, PIP_3 produced upon growth factor stimulation is responsible for recruiting PKB to the plasma membrane, where it is phosphorylated by PDK1 at its activation loop on T308 (Alessi et al., 1997; Andjelkovic et al., 1997). In order for PKB to perform its kinase activity, it has to be further phosphorylated on S473 at the hydrophobic motif by mTORC2, and this phosphorylation is essential for PKB activation (Sarbassov et al., 2005). Furthermore, mTORC2 is also responsible for phosphorylating PKB on T450 at the turn motif (Oh et al., 2010). In short, mTORC2 phosphorylates PKB on S473 and T450 to elicit its full activation, and hence, PKB can effectively stimulate its substrates to regulate numerous cellular functions. For instance, FoxOs (transcription factors of the Forkhead box O class) are a family of transcription factors which promote the transcription of cell cycle inhibitors, and factors that induce apoptosis (Dijkers et al., 2000a, 2000b). Upon their phosphorylation by PKB, FoxOs are inhibited and hence, cell proliferation and survival are enhanced (Kloet and Burgering, 2011). Moreover, PKB also promotes cell survival with the aid of 14-3-3 protein. When exposed to survival factors, PKB phosphorylates BAD, a proapoptotic Bcl-2 family protein, on S136 and this phosphorylation leads to the association of 14-3-3 protein with BAD. As such, the accessibility of kinases, like PKB, to phosphorylate BAD on S155 is greatly enhanced and such phosphorylation inhibits BAD from interacting with prosurvival Bcl-2 family members to induce apoptosis (Datta et al., 1997, 2000). PKB also upregulates protein synthesis by phosphorylating and inhibiting TSC2 and PRAS40, leading to the activation of mTORC1 signaling that enhances protein synthesis via S6K1 and 4E-BP1. Furthermore, PKB also modulates the activity of enzymes involved in

metabolism. For example, PKB has been shown to induce the localization of hexokinases to mitochondria, a process that can directly couple glucose metabolism to oxidative phosphorylation via yet-to-be defined mediator(s) (Gottlob et al., 2001). As a wide range of cellular physiology is mediated by PKB, it is not unexpected that dysregulation of PKB as well as its kinase mTORC2 are found to be involved in a variety of pathological conditions including cancers and diabetes (Hers et al., 2011; Oh and Jacinto, 2011). PKB has been localized to the BTB and apical ES in the seminiferous epithelium of rat testes, and its expression at these sites was found to be stage-specific, being highest at stage VI–VII but considerably diminished by early stage VIII and further diminished by late stage VIII of the epithelial cycle when BTB restructuring and apical ES degeneration take place to facilitate preleptotene spermatocyte migration and spermiation at the corresponding site (Siu et al., 2005). It is noted that this pattern of stage-specific expression of PKB at the apical ES is somewhat similar to the stage-specific expression of p-rpS6 at the apical ES (Mok et al., 2012c), illustrating PKB and rpS6 can be the downstream signaling molecules and substrates of mTORC2 and mTORC1, respectively, that mediate cross talk between the two mTOR signaling complexes.

3.3.2.2. Protein Kinase C-α

Unlike the other two mTORC2 effectors PKB and SGK1, which are substrates of mTORC2, it remains unclear whether PKC-α is directly phosphorylated by mTORC2 or through other mediator(s) (Sarbassov et al., 2004). However, after the knockdown of rictor by RNAi, phosphorylation of PKC-α on S657 was shown to be reduced, resulting in the change of cell shape due to actin reorganization in which actin filaments at the cortical sides became less prominent and stress fibers were formed in the cytosol. Similar morphology of actin cytoskeleton was observed after PKC-α knockdown, validating actin organization is indeed regulated by mTORC2 and is mediated through PKC-α (Sarbassov et al., 2004). In addition to that, a recent study showed that RNAi-mediated knockdown of rictor in cultured Sertoli cells also led to a reduced PKC-α phosphorylation, which in turn resulted in actin reorganization (Mok et al., 2012a). Furthermore, addition of serum to serum-starved fibroblasts induced rapid and robust stress-fiber formation, which was ablated by a knockdown of mTORC2 subunits mTOR, mLST8 and rictor (Jacinto et al., 2004). Furthermore, during the actin cytoskeleton restructuring due to the knockdown of mTORC2 subunits, a decline in GTP-bound Rac1 was observed. Whereas cells overexpressing constitutively active form of Rac1 and Rho were able to resist actin reorganization due to

reduced mTORC2, this thus suggests that small GTPase Rac1 and Rho are possible mediators of mTORC2 in controlling actin organization (Jacinto et al., 2004). In fact, these small GTPases are likely downstream molecules of PKC-α (Fig. 6.3). This notion was supported by studies revealing that the Rho-induced actin rearrangement, such as stress-fibers formation, in endothelial cells was PKC-α-dependent. It was shown that upon treatment of TNF-α or thrombin, actin reorganization was induced and such actin reorganization was found to be mediated by activation of Rho GTPase activator p115RhoGEF (a guanine nucleotide exchange factor for Rho GTPase) by PKC-α (Holinstat et al., 2003; Peng et al., 2011). Moreover, it is known that PKC-α is also able to activate Rho by negatively regulating the Rho inhibitor, Rho-GDP guanine nucleotide dissociation inhibitor (GDI), via phosphorylation (Mehta et al., 2001). In short, the above studies suggest that small GTPases, such as Rho and Rac1, are downstream molecules of PKC-α in the mTORC2 signaling pathway that regulates actin cytoskeleton.

3.3.2.3. Serum- and Glucocorticoid-induced Protein Kinase 1 (SGK1)

SGK1 is an important substrate of mTORC2. Similar to PKB, following insulin and growth factor stimulation, SGK1 is phosphorylated by PDK1 at its activation loop on T256 (Biondi, 2004; Mora et al., 2004). In order to fully activate SGK1, mTORC2 is responsible for phosphorylating its S422 residue at the hydrophobic motif (Garcia-Martinez and Alessi, 2008). More important, it has been shown that in fibroblasts lacking mTORC2 subunits rictor, Sin1 or mLST8, the phosphorylation of SGK1 as well as its substrate N-myc downstream regulated gene 1 (NDRG1) is abrogated, illustrating the necessity of the functional mTORC2 in activating SGK1. SGK1 is also a Ser/Thr kinase that regulates a variety of cellular functions including ion transport, cell proliferation and survival (Garcia-Martinez and Alessi, 2008). Studies have shown that FoxOs are transcription factors that promote gene transcription involving in induction of apoptosis and inhibition of cell cycle progression (Dijkers et al., 2000a, 2000b), SGK1 was found to stimulate cell survival and proliferation by directly phosphorylating FoxO3a to inhibit its nuclear localization and transcription activity (Brunet et al., 2001; Dehner et al., 2008). Moreover, given the fact that p53 is able to trigger apoptosis (Soengas et al., 1999), SGK1 also enhances cell survival by phosphorylating an E3 ubiquitin ligase Mdm2 (murine double minute, an oncogene) on S166 to promote its binding to p53 for ubiquitination (Amato et al., 2009). Furthermore, mTORC2/SGK1 signaling is required for the expression of another p53 E3 ubiquitin ligase called human double minute 2 (HDM2). A knockdown of rictor or SGK1 by RNAi

led to downregulation of HDM2, illustrating the significant of mTORC2/SGK1 pathway for inhibiting p53 to modulate cell survival (Lyo et al., 2010). Taken collectively, these studies show that besides PKB, mTORC2 can modulate cell proliferation as well as survival through SGK1. SGK1 is likely to play a crucial role in carcinogenesis since it is overexpressed in a variety of tumors (Fagerli et al., 2011; Simon et al., 2007; Zhang et al., 2005) and its suppression is able to reduce tumor development. For instance, mice lacking SGK1 were found to be more resistant to chemically induced colon cancer (Nasir et al., 2009). Whereas in adenoma polyposis coli (APC)-deficient mice, which developed gastrointestinal tumors spontaneously, the lack of SGK1 led to reduced intestinal tumor development (Wang et al., 2010). However, the role of SGK1 in spermatogenesis and other testicular function remain unexplored. Nontheless, these findings illustrate that SGK1 may be involved in regulating germ cell apoptosis during spermatogenesis.

3.4. The Interplay between mTORC1 and mTORC2 in Regulating Cellular Events

As described above, mTORC1 and mTORC2 have their distinctive downstream substrates and signaling molecules so that they regulate distinctive cellular functions. However, these two pathways are also interconnected and can interact with each other to affect phenotypes. For example, both signaling complexes are activated upon stimulation by growth factors and amino acids. Besides, they also share the same upstream regulator, TSC1/2 complex, which promotes the activity of mTORC1 but suppresses mTORC2 (Fig. 6.3). More important, S6K1, which is the substrate of mTORC1, can phosphorylate rictor, the critical binding partner of mTORC2, and inhibit the catalytic activity of mTORC2 on PKB, which is also the upstream regulator of mTORC1, thereby creating as a negative feedback loop (Fig. 6.3). Besides sharing common activating stimuli and regulators, recent studies have suggested that some of the cellular functions modulated by these signaling complexes are indeed overlapping, despite the fact that they have their specific substrates. For instance, mTORC1 regulates cell proliferation via S6K1 and rpS6, whereas mTORC2 modulates the same cellular process with PKB and SGK1. Furthermore, regulation of actin cytoskeleton was once regarded as a specific role of mTORC2, but several recent studies indicate that mTORC1 may be involved in this event. First, a study performed in yeasts revealed that rapamycin treatment which inhibited TORC1 signaling was found to perturb actin polarization within 10 min, and this treatment also delayed actin repolarization after glucose starvation (Aronova et al., 2007). Since significant actin depolarization

was determined in such a short interval (within 10 min) after adding rapamycin, the actin reorganization should be attributed to a loss of TOR1 function *only* since mTORC2 remained unaffected during this short period of time (Aronova et al., 2007). Second, in Rh30 and dU-373 mammalian cancer cell lines, treatment of these cells with rapamycin for 2 h was found to inhibit the type I insulin-like growth factor (IGF-I)-stimulated F-actin reorganization, confirming the involvement of mTORC1 signaling in actin dynamics (Liu et al., 2008). Also, in ovarian cancer cells transfected with constitutively active S6K1, actin reorganization to facilitate the formation of actin-based lamellipodia, actin microspikes and filopodia were induced in these cells, and such actin cytoskeleton restructuring was mediated via Rac1 and Cdc42 (Ip et al., 2011). Furthermore, phosphorylated S6K1 was found to bind to F-actin, cross-linking actin filaments, thereby stabilizing F-actin as it significantly reduced the rate and extent of actin filament depolymerization induced by cofilin (Ip et al., 2011). In short, these recent findings illustrate that although mTORC1 and mTORC2 possess distinctive substrates and different downstream signaling molecules, they both regulate cell proliferation and F-actin organization in cells.

3.5. Regulation of Blood–Tissue Barrier Function by mTOR

3.5.1. Regulation of Barrier Function in The Kidney by mTOR

Among the numerous cellular processes mediated by mTOR, its effects on immune response in mammals are well characterized. Rapamycin, a potent inhibitor of mTOR, is an immunosuppressant drug widely used by kidney and heart transplant patients (Diekmann and Campistol, 2006; Kahan, 2001). However, after prolonged exposure to rapamycin, proteinuria (a pathological condition with excessive serum proteins found in urine) and even nephritic syndrome were observed in some patients (Aliabadi et al., 2008; Dittrich et al., 2004; Izzedine et al., 2005; van den Akker et al., 2006). Such pathological condition was later found to be the result of damages in podocytes, which are the cells responsible for maintaining the blood–urine filtration barrier of the renal glomerulus in the kidney. This selective barrier is created via a unique cell–cell contact called the slit diaphragm established by primary and secondary foot processes from podocytes (Paventadt et al., 2003). In cultured human immortal podocytes, prolonged treatment of rapamycin downregulated mTOR and rictor and thus reduced the formation of mTORC2, leading to reduced phosphorylation of PKB on S473 (Vollenbroker et al., 2009). The suppression of mTORC2 signaling disrupted the podocyte-based filtration barrier, which was the result of reduced cell adhesion. Such reduction of cell adhesion was mediated, at least in part, by a loss of slit diaphragm proteins,

such as nephrin, and a reorganization of actin cytoskeleton. It was observed that formation of dot-like actin-rich structures were enhanced by rapamycin, and this actin reorganization was caused by a loss of Nck (non-catalytic region of tyrosine kinase adaptor protein 1), which is an actin regulating protein and a cytoskeleton adaptor that links nephrin to actin cytoskeleton (Vollenbroker et al., 2009). Besides long-term rapamycin treatment, diabetes also leads to malfunction of blood–urine filtration barrier, resulting in proteinuria. It was demonstrated that diabetes led to overactivation of mTOR signaling in damaged podocytes in diabetic mice, leading to mislocalization of slit diaphragm protein nephrin and also TJ adaptor ZO-1, moving from plasma membrane to cytosol (Inoki et al., 2011). The fact that the phenotypes of podocyte damages found in diabetic animals mimicked podocyte-specific TSC1 knockout mice (note: TSC1 is the mTORC1 upstream negative regulator, see Fig. 6.3), illustrating the involvement of mTORC1 signaling in the podocyte-based filtration barrier. The role of mTORC1 and mTORC2 in regulating the blood–urine filtration barrier was also illustrated in a study using podocyte-specific raptor or rictor knockout mice (Godel et al., 2011). Mice lacking mTORC1 in podocytes as the result of podocyte-specific raptor knockout developed significant albuminuria, a form of proteinuria. In contrast, loss of mTORC1 in podocytes of adult mice triggered by conditional knockout of raptor only had a mild effect and the level of protein excreted in urine in these mice was insignificantly higher than that of the wild-type (Godel et al., 2011). Additionally, it was shown that when conditional knockout of raptor was performed in mice with genetic background that was known to be more sensitive toward podocyte damage, significant proteinuria was induced (Godel et al., 2011). Taken together, these findings illustrate that mTORC1 signaling is required for proper development of podocytes to form the blood–urine filtration barrier; whereas in adult mice after podocytes are developed and the blood–urine filtration barrier is fully functional, mTORC1 is necessary for maintenance of podocyte functions, and mTORC1 is more important in animals with specific genetic background. It is noted that while podocytes are needed mTORC1 to maintain the filtration barrier function, overactivation of mTORC1 signaling in podocytes also leads to a disruption of the barrier. This indicates that a precise control on the availability of mTORC1 is needed to maintain the homeostasis of the barrier function. Regarding the role of mTORC2 in podocyte-mediated barrier function, it was shown that in podocyte-specific rictor knockout mice, only transient albuminuria was found when these mice were challenged by a BSA overload (Godel et al., 2011). However, when raptor and rictor were simultaneously knockout in podocytes, massive proteinuria

was observed, suggesting mTORC2 signaling is necessary for podocytes to cope with stress conditions and both mTOR complexes work synergistically together to maintain the integrity of the filtration barrier in the kidney.

It was known that induction of mTORC1 activity by simultaneous deletion of PTEN and Lkb1, two negative upstream regulators of mTORC1 (Fig. 6.3), in mouse bladder epithelial cells led to a loss of AJ protein E-cadherin and TJ adaptor ZO-1, leading to tumor progression (Shorning et al., 2011). Moreover, it was reported that a knockdown of rictor by RNAi in glioma cells led to induction of matrix metalloproteinase-9 (MMP-9) mediated by activation of Raf-1-MEK-ERK pathway, and such activation was caused by the removal of the inhibitory effect from PKB due to a loss of mTORC2 function. Since MMP-9 is responsible for breaking down extracellular matrix via its action on collagen IV, its induction thus contributes to an increase in invasiveness of glioma tumor cells (Das et al., 2011). In addition, it was shown that in cultured Sertoli cells, an induction of MMP-9, such as by TNFα, that led to a disruption of the TJ barrier was mediated via a downregulation of TJ protein occludin (Siu et al., 2003). Collectively, these findings suggest that in Sertoli cells, suppression of mTORC2 activity may result in an MMP-9-mediated disruption of the BTB. In fact, a recent study has shown that a reduced mTORC2 activity perturbs the Sertoli BTB function (Mok et al., 2012a), whereas a reduced mTORC1 signaling function promotes the Sertoli TJ-permeability barrier (Mok et al., 2012c). These findings thus suggest that these two mTOR complexes work antagonistically to modulate BTB dynamics in the testis.

4. REGULATION OF BTB DYNAMICS BY mTOR

4.1. Background

The involvement of mTOR in BTB dynamics during spermatogenesis has not been explored until recently (Mok et al., 2012a; Mok et al., 2012c). As shown in Fig. 6.4, both mTOR and the crucial subunits that create mTORC1 (e.g. raptor) and mTORC2 (e.g. rictor) were localized in the seminiferous epithelium near the basement membrane, consistent with their localization at the BTB. However, it is noted that the stage-specific expression of raptor and rictor during the epithelial cycle is different, with raptor being the highest, but rictor at its lowest, at stage IX of the epithelial cycle (Fig. 6.4), implicating the mTORC1 and mTORC2 may have differential effects on the BTB. These recent findings (Mok et al., 2012a; Mok et al., 2012c) (Fig. 6.4) coupled with results of other studies in the field thus support a novel concept depicted in Fig. 6.5 regarding the "yin" and

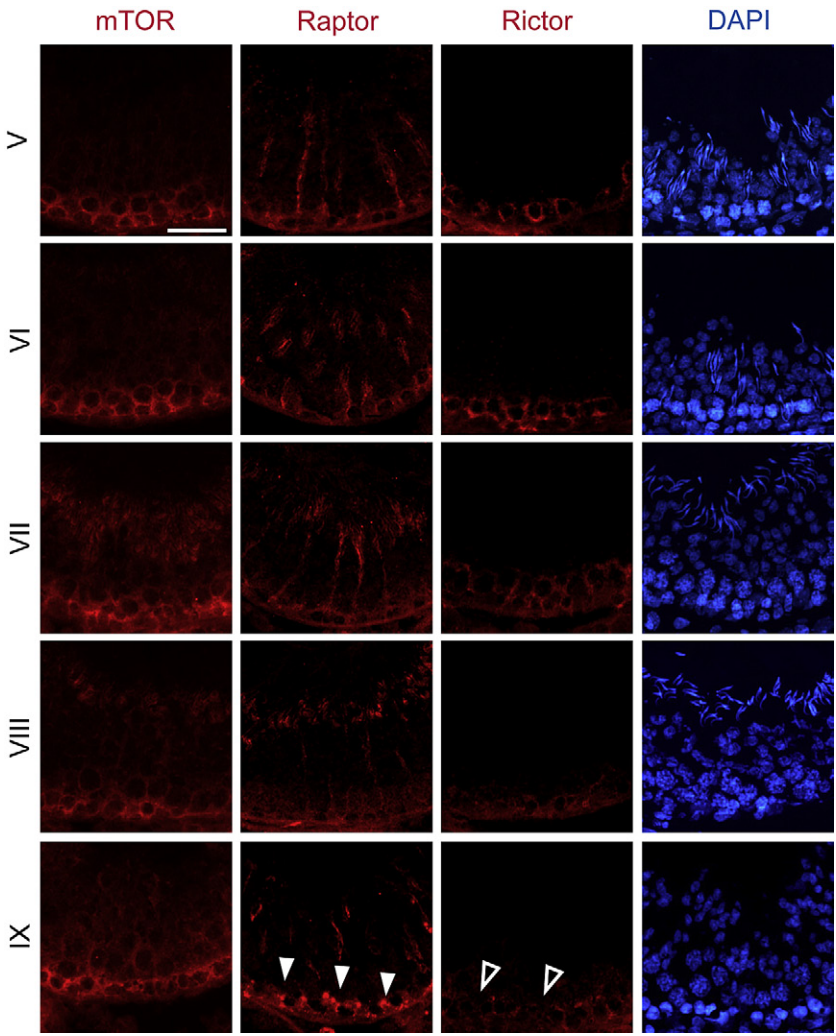

Figure 6.4 ***Stage-specific expression of raptor and rictor versus mTOR in the seminiferous epithelium of adult rat testes.*** Relative expression level and localization of mTOR, raptor and rictor (red) in the seminiferous epithelium from stage V–IX tubules were examined by immunofluorescence microscopy. Cell nuclei were stained with DAPI (blue) to show the stages of the tubules. This figure shows that from stage V to IX, mTOR was expressed at relatively similar level at the basal compartment where BTB was located. On the other hand, the expression of raptor, which is the key binding partner of mTORC1, was transiently induced at stage IX (indicated by white arrow head) that begin in late stage VIII, most notably at the BTB, whereas the expression of rictor, which is the key subunit of mTORC2, was found to decline gradually from stage VII and became barely detectable at late stage VIII through stage IX (indicated by open arrow head), even though it remained weakly expressed in these stages. Bar, 50 μm, which applies to all micrograph. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

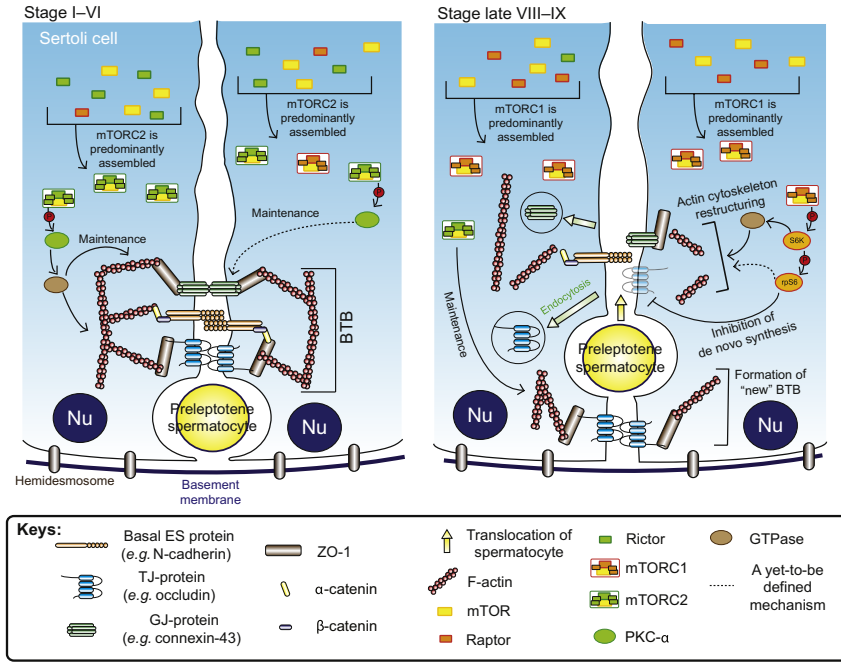

Figure 6.5 ***mTORC1 and mTORC2 display antagonistic effects on the BTB and their combined effects can protect the immunological barrier integrity during the transit of preleptotene spermatocytes at the BTB.*** It is noted that rictor and raptor compete for mTOR for the formation of mTORC2 and mTORC1, respectively, to promote BTB and disrupt BTB integrity. At stages I–VI in which prior to BTB restructuring, the relative high expression of rictor favors the assembly of mTORC2, which is necessary for keeping the integrity of BTB by maintaining the dense F-actin network (namely the actin filament bundles at the basal ES), expression level of GJ proteins and GJ communication. On the other hand, in stage late VIII to stage IX that the BTB is transiently "open" to facilitate the transit of spermatocyte, the expression level of raptor is induced, whereas that of rictor is reduced. Thus, formation of mTORC2 is reduced and mTORC1 is favored. mTORC1 activates rpS6, which in turn disrupts the "old" BTB above the preleptotene spermatocytes in transit at the BTB by inhibiting de novo synthesis of BTB proteins. In addition, actin cytoskeleton reorganization during BTB restructuring is induced by (i) mTORC1 signaling via S6K1 and rpS6 and (ii) the decrease in phosphorylated PKC-α due to reduced mTORC2. The disorganized F-actin network leads to internalization of BTB proteins, which perturbs the "old" BTB. Furthermore, the decrease in GJ proteins and GJ communication caused by reduced mTORC2 also facilitates the disruption of the "old" BTB for the translocation of spermatocytes across the BTB. However, while mTORC2 expression is reduced, it remains to be robust enough to sustain the maintenance of the "new" BTB that is being assembled behind the preleptotene spermatocytes in transit. In short, utilizing the antagonistic effects of the mTORC1 and mTORC2 on the TJ-permeability barrier, the immunological barrier function can be maintained during the passage of preleptotene spermatocytes, which are connected in "clones" via intercellular bridges, at the BTB. For color version of this figure, the reader is referred to the online version of this book.

"yang" effects of the mTORC1 and mTORC2 signaling complexes on the BTB dynamics that regulate BTB restructuring during the seminiferous epithelial cycle of spermatogenesis, which is being critically evaluated in the following sections.

4.2. Regulation of BTB Dynamics by mTORC1

In the seminiferous epithelium of adult rat testes, rpS6, a crucial downstream signaling molecule of mTORC1 (Section 3.2.2.) was found to be highly expressed in the basal compartment of the seminiferous epithelium in all stages of the epithelial cycle, consistent with its localization at the BTB, implicating the likely involvement of mTORC1 signaling complex in BTB dynamics (Mok et al., 2012c). Interestingly, p-rpS6, the activated form of rpS6, was highly expressed at the BTB and colocalized with putative BTB proteins ZO-1, N-cadherin and Arp3, but restrictive to late stage VIII–IX, coinciding with the time of BTB restructuring to facilitate the transit of preleptotene spermatocytes at the site (Mok et al., 2012c). This timely upregulation in the phosphorylated and activated form of rpS6 at the BTB suggests that rpS6 may take part in the "opening" of the BTB for the transit of spermatocytes from the basal to the apical compartment. To confirm this postulate, rpS6 phosphorylation was abolished by inactivating mTORC1 signaling in cultured Sertoli cells with an established TJ-permeability barrier by either treatment of cells with rapamycin or a knockdown of rpS6 by RNAi, both approaches was shown to promote the Sertoli cell TJ barrier by making the BTB "tighter" following a blockade rpS6 activation or its knockdown (Mok et al., 2012c). In addition, the expression of TJ proteins, such as claudin-11, were upregulated with claudin-11 being redistributed and localized more intensely to the Sertoli cell–cell interface (Mok et al., 2012c), possibly being used to "strengthen" the TJ barrier. Furthermore, changes in the F-actin organization was detected with more actin filaments were found at the Sertoli cell–cell interface (Mok et al., 2012c), possibly being used to strengthen the Sertoli cell TJ barrier. In short, these findings illustrate that rpS6 was specifically activated and highly expressed at the site of the BTB in the seminiferous epithelium during its restructuring at stage VIII–IX of the epithelial cycle, whereas a suppression of rpS6 or its knockdown in Sertoli cells led to a "tightening" of the TJ barrier. These findings thus support the notion that the rpS6 activation is crucial to elicit BTB restructuring, such as at stage VIII–IX of the epithelial cycle. An earlier study has shown that mouse embryonic fibroblasts (MEFs, also known as feeder

cells) from rpS6$^{p-/-}$ mice displayed a higher rate of global protein synthesis (Ruvinsky and Meyuhas, 2006), suggesting that a decline in phosphorylated rpS6 might trigger de novo synthesis of TJ proteins, which is consistent with our findings that a knockdown of rpS6 in Sertoli cell epithelium induced claudin-11 expression (Mok et al., 2012c). Furthermore, rpS6 might take part in regulating actin cytoskeleton similar to its upstream activator S6K1 since actin filament rearrangement was shown to be stimulated following a knockdown of rpS6; and to further support the role of rpS6 in actin dynamics, phosphorylated rpS6 was found to structurally interact with actin as demonstrated by coimmunoprecipitation (Mok et al., 2012c). Taking these findings collectively, it is clear that the promotion of the Sertoli cell TJ-barrier function after a suppression of rpS6 likely leads to an increase in the synthesis of TJ proteins (e.g. claudin-11), which coupled with redistribution and/or relocalization of BTB proteins to the Sertoli cell–cell interface, supported by an increase in F-actin bundles at the cortical region of the Sertoli cells in the epithelium, thereby strengthening the BTB integrity. In short, during the epithelial cycle of spermatogenesis, the timely activation of mTORC1 at stage VIII–IX that leads to phosphorylation of rpS6 during BTB restructuring may facilitate this process by transiently downregulating TJ proteins, and perturbing the supportive F-actin network underneath cell adhesion complexes that facilitates their endocytosis. In short, BTB is transiently "opened" above the preleptotene spermatocytes in transit at the BTB induced by an upregulation of p-rpS6, which facilitates the migration of these spermatocytes across the BTB to enter the adluminal compartment to prepare for meiosis I/II.

4.3. Regulation of BTB Dynamics by mTORC2

For mTORC2, its key binding partner rictor was shown to be highly expressed at the BTB from stages I–VI of the seminiferous epithelial cycle, however, it was downregulated from late stage VII and it was considerably diminished and barely detectable at stage IX (Mok et al., 2012a) (Fig. 6.4). This suggests that mTORC2 signaling may be involved in maintaining the BTB integrity during all the stages of the epithelial cycle of spermatogenesis except at stage VIII–IX when it is downregulated when the BTB is under restructuring (Mok et al., 2012a). To confirm this postulate, studies were performed in which a knockdown of rictor by RNAi in cultured Sertoli cells with an established TJ-permeability barrier was found to disrupt the TJ barrier, and this event was also associated with a reduced phosphorylation of PKC-α, but not PKB (Mok et al., 2012a). Thus, the Raf-1-MEK-ERK

pathway, which is inhibited by PKB, was not activated and the level of MMP-9 remained unchanged (Mok et al., 2012a). As discussed in Section 3.2.1, mTORC2 signaling complex regulates actin cytoskeleton via PKC-α in multiple epithelia; thus, the knockdown of rictor by RNAi triggered actin reorganization, and actin filaments were rearranged in Sertoli cells with reduced F-actin to support the TJ-barrier function at the Sertoli cell–cell interface (Mok et al., 2012a). Interestingly, following the rictor knockdown in Sertoli cells by RNAi that led to a reduction in phosphorylated PKC-α, the expression of Cx26 and Cx43 in these Sertoli cells was also downregulated (Mok et al., 2012a). Furthermore, TJ proteins occludin and ZO-1 were also redistributed from the cell–cell interface and moved into the cell cytosol (Mok et al., 2012a), thereby destabilizing cell adhesion, leading to the Sertoli cell TJ-barrier disruption. These findings thus illustrate that a knockdown of rictor in Sertoli cells leads to restructuring of actin cytoskeleton, reducing cortical F-actin, this thus facilitates internalization of TJ proteins and hence weakening the TJ barrier. More important, it was demonstrated that a knockdown of rictor led to a disruption of GJ communication between adjacent Sertoli cells based on a functional GJ-channel assay (Mok et al., 2012a). Collectively, these findings thus support the notion that during the seminiferous epithelial cycle of spermatogenesis, rictor and, hence, mTORC2 signaling is essential for maintaining BTB integrity. When rictor is downregulated during the epithelial cycle, such as at stage VIII at the time of BTB restructuring, this leads to PKC-α-mediated actin cytoskeleton reorganization that promotes endocytosis of TJ proteins to destabilize the BTB above the preleptotene spermatocytes in transient at the BTB. This process is also assisted by a downregulation of GJ proteins, which coordinates with the timely "disassembly" of TJ and basal ES at the site to facilitate the transit of spermatocytes.

4.4. A Hypothetic Model Based on The Antagonistic Effects of mTORC1 and mTORC2 on BTB Function to Regulate its Integrity during The Epithelial Cycle of Spermatogenesis

Based on recent findings as discussed above, it is clear that the action of mTORC1 is to promote the "disassembly" of the BTB while mTORC2 supports BTB integrity. It is very likely that the simultaneous presence of these two signaling complexes in the seminiferous epithelium that exert their antagonistic effects on the underlying actin cytoskeleton at the BTB that leads to changes in the localization of TJ proteins play a critical role in maintaining the BTB integrity during the transit of preleptotene spermatocytes,

which are connected in "clones," at the BTB. Figure 6.5 depicts a hypothetical model regarding the involvement of mTORC1 and mTORC2 in regulating BTB integrity during the epithelial cycle of spermatogenesis. It is hypothesized that during the epithelial cycle, upregulation of rictor at stages I–VII that favors the formation of mTORC2 is being used to maintain the BTB integrity, but not at stages VIII–IX when its expression is downregulated at the time of BTB restructuring. On the other hand, during stage late VIII–IX, the transient-induced expression of raptor favors the formation of mTORC1 for the disruption of the "old" BTB at the apical region of the transiting preleptotene spermatocytes at the site. This process is further facilitated by the reduction in mTORC2 due to a downregulation of rictor (Figs 6.4 and 6.5). Furthermore, the low level of rictor expressed during the BTB restructuring may be necessary for the "assembly" and "maintenance" of the "new" BTB that is being created at the basal region of the transiting preleptotene spermatocytes (Fig. 6.5). In fact, the dependence of relative abundance of raptor and rictor for the activation of mTORC1 or mTORC2 signaling has been demonstrated in other studies. For example, it was reported that the knockdown of raptor by RNAi in HEK-293T and HeLa cells led to an increase in PKB phosphorylation on S473, indicating mTORC2 signaling was enhanced. On the other hand, in the same cell types, knockdown of rictor caused increased phosphorylation of S6K1 with increased association between raptor and mTOR, revealing mTORC1 signaling was stimulated (Sarbassov et al., 2004). More important, it was revealed that in mTOR-mediated mitochondrial metabolism, a knockdown of raptor reduced oxygen consumption while a knockdown of rictor increased oxygen consumption and oxidative capacity (Schieke et al., 2006). These studies thus illustrate how a cellular function can be modulated based on the "yin-and-yang" effects of the two mTOC complexes mediated by the relative availability of raptor and rictor in a cellular microenvironment. In short, the combined antagonistic effects of the mTORC1 or mTORC2 signaling complexes can fine-tune a cellular event, such as the migration of preleptotene spermatocytes across the BTB as depicted in Fig. 6.5.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this chapter, we have provided a critical update on the biology of adhesion junctions as well as the role of constituent proteins in regulating BTB dynamics in the testis. We have also reviewed the functional relationship

between these proteins and the underlying actin cytoskeleton. While some of the discussions are based on findings in other epithelia/endothelia, this information will be helpful to design functional experiments in future studies to unravel the regulation of the BTB. We also provide an update on the latest development regarding the involvement of the two mTOR signaling complexes, namely mTORC1 and mTORC2, in regulating BTB dynamics during the seminiferous epithelial cycle of spermatogenesis. Although recent studies have shown that the mTORC1 and mTORC2 signaling complexes likely modulate BTB dynamics their antagonistic effects on the TJ-permeability barrier function via actin cytoskeleton, however, the actin regulatory proteins involved in these events remain to be identified and examined. Much work is needed to explore if mTOR complexes exert their effects on the F-actin via drebrin E, paladin, formins, filamins, Eps8, the Arp2/3 complex and others. Other small GTPases such as Rac and Rho and polarity proteins (e.g. PAR3, PAR6, 14-3-3, Scribble/Dlg/Lgl) may also be involved. Moreover, the molecular mechanism(s) by which rictor regulates the expression of GJ proteins and GJ communication, which in turn modulates BTB dynamics, remains to be identified. Additionally, we hypothesize that mTORC1 and mTORC2 regulate BTB dynamics via their antagonistic effects on BTB assembly and maintenance, and the activity of these two signal complexes are mediated by the relative expression of their key binding partners raptor and rictor and downstream signaling molecules, such as rpS6, in the seminiferous epithelium. While much work is needed, however, the model depicted in Fig. 6.5 provides a framework upon which functional studies can be designed to understand the interplay between mTOR complexes and other regulatory proteins that modulate the BTB function during spermatogenesis.

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CHAPTER SEVEN

New Approaches for the Identification of Drug Targets in Protozoan Parasites

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Abstract

Antiparasitic chemotherapy is an important issue for drug development. Traditionally, novel compounds with antiprotozoan activities have been identified by screening of compound libraries in high-throughput systems. More recently developed approaches employ target-based drug design supported by genomics and proteomics of protozoan parasites. In this chapter, the drug targets in protozoan parasites are reviewed. The gene-expression machinery has been among the first targets for antiparasitic drugs and is still under investigation as a target for novel compounds. Other targets include cytoskeletal proteins, proteins involved in intracellular signaling, membranes, and enzymes participating in intermediary metabolism. In apicomplexan parasites, the apicoplast is a suitable target for established and novel drugs. Some drugs act on multiple subcellular targets. Drugs with nitro groups generate free radicals under anaerobic growth conditions, and drugs with peroxide groups generate radicals under aerobic growth conditions, both affecting multiple cellular pathways. Mefloquine and thiazolides are presented as examples for antiprotozoan compounds with multiple (side) effects. The classic approach of drug discovery employing high-throughput physiological screenings followed by identification of drug targets has yielded the mainstream of current antiprotozoal drugs. Target-based drug design supported by genomics and proteomics of protozoan parasites has not produced any antiparasitic drug so far. The reason for this is discussed and a synthesis of both methods is proposed.

1. INTRODUCTION

Protozoan parasites have constituted serious challenges to public health since the beginning of mankind. Sustainable protection can only be achieved by a combination of improved sanitary conditions, vaccination (Chilengi and Gitaka, 2010; Innes and Vermeulen, 2007), and chemotherapy. During the past decades, novel anti-infective drugs have regained importance because of the steady increase in resistance development against well-established antibiotics and due to the emergence of novel, previously unnoticed infectious diseases (Cassell and Mekalanos, 2001). This does account not only for bacterial and viral diseases but also for infections caused by protozoans, especially *Toxoplasma*, *Giardia* and *Plasmodium* (Egan and Kaschula, 2007). Currently, only a limited number of drugs are available on the market to confront a plethora of parasites, and in case of resistance development, the arsenal of alternative treatment options is limited (Table 7.1). *Giardia lamblia*, responsible for several million cases of persistent diarrhea worldwide, is currently treated almost exclusively with metronidazole (Gardner and Hill, 2001). The

Table 7.1 Overview of the most prominent diseases caused by protozoa and drugs that are currently used for chemotherapy and their approximate time of introduction. Sources: WHO (www.who.int), NIH (www.nlm.nih.gov)

Disease	Causative agent	Estimated cases	Drugs	Introduction
Malaria	*Plasmodium falciparum*	250 ($\times 10^6$/year)	Quinine	Seventeenth century
			Quinacrine	1930
			Chloroquine	Around 1945
			Mefloquine	1985
			Artemisinine and derivatives	1972
			Doxycycline	<1990
			Proguanil (+Atovaquone)	1946
Giardiasis	*Giardia lamblia*	200	Quinacrine	1941
			Metronidazole, tinidazole	1961
			Furazolidone	>1970
			Nitazoxanide	1996
			Paromomycine	1960
Leishmaniasis	*Leishmania* sp.	1–2	Pentamidine	1930
			Liposomal amphotericin B	1990
			Antimony-(V)-compounds	1950
African trypanosomiasis (sleeping sickness)	*Trypanosoma brucei*	0.01	Pentamidine	1930
			Nifurtimox–eflornithine	2009
Chagas disease	*Trypanosoma cruzi*	Ten currently infected (chronic disease)	Benznidazole together with nifurtimox	1980

apicomplexan *Toxoplasma gondii* infects an estimated 25% of the population, and thus is one of the most successful parasites worldwide (Innes and Vermeulen, 2007). While pregnant women and immunocompromised persons represent the main risk groups, there is an increasing number of reports concerning clinical toxoplasmosis in immunocompetent individuals (McAllister, 2005) where up to two-thirds of infections cannot be explained by risk factors such as consuming raw or undercooked meat or poor kitchen hygiene (Petersen et al., 2010). The current treatment options for toxoplasmosis are limited and include only few compounds such as pyrimethamine,

clindamycin or trimethoprim alone or in combination with sulfonamides (Greif et al., 2001). These drugs are not specific for toxoplasmosis, they do not notably affect the tissue cyst stage, and may have side effects, mainly in pregnant women (Innes and Vermeulen, 2007; Petersen et al., 2010). These few examples show that novel and more specific drugs are needed to supplement the arsenal of antiparasitic compounds. In principle, drug development occurs by two complementary approaches, namely whole organism screening (Cos et al., 2006) and drug target-based development (Seib et al., 2009). In any case, the drug targets have to be validated in a multistep process (Egner et al., 2005). The screening for inhibitors derived from natural compounds or from libraries of *de novo* synthesized compounds based on existing chemical scaffolds provides still a major source of drug candidates (Kayser et al., 2003). Currently, an increasing effort in drug design based on genomics and proteomics is made but it is unclear to which extent it will replace classical drug screening (Timmers et al., 2009). In order to provide a rationale for the mode of action of a given compound, in each case, the drug target has to be identified. Here, we present a review on recent approaches for the identification of drug targets in protozoan parasites.

2. STRATEGIES FOR DRUG TARGET IDENTIFICATION

2.1. Evaluation of Drug Effectiveness

2.1.1. Screening Systems

The starting point of each whole organism-based screening for antiparasitic compounds is the setup of a suitable system. Since in vivo tests are expensive and are limited for ethical reasons, in vitro systems have replaced in vivo tests as a first step of drug development. A panel of suitable in vitro methods for antiparasitic drug screening is given in Table 7.2.

For monitoring the effects on parasite proliferation, morphological assays represent the gold standard. In the case of axenically grown protozoa (e.g. *G. lamblia*), cultures are treated with the compounds to be tested, and after a given time period, the live parasites are counted under the microscope (Müller et al., 2006). This provides information not only on growth inhibition but also on effects on shape and mobility of the parasite. In the case of obligate biotrophic parasites grown in co-culture with host cells, parasite-specific structures such as intracellular cysts are visualized by selected staining methods (Müller et al., 2009a) prior to counting. All

Table 7.2 Selected examples of in-vitro-screening methods for antiprotozoal compounds

Principle	Method	Organism	Reference
Counting by eye	Staining of infected cells with cresyl violet. Counting of cysts.	*Toxoplasma gondii*	(Müller et al., 2009a)
	Counting of trophozoites under the microscope.	*Giardia lamblia*	(Müller et al., 2006)
Quantification by dyes	Staining with methylene blue. Colorimetric assay.	*Giardia lamblia*	(Busatti and Gomes, 2007)
	Staining of parasite DNA in human erythrocytes by intercalating dye.	*Plasmodium falciparum*	(Banieck et al., 2007) (Duffy and Avery, 2012)
	Immunofluorescence of liver stages in infected hepatocytes.	*Plasmodium* sp.	(Gego et al., 2006)
Quantification after radioactive labeling	Incorporation of radioactive precursor into DNA, counting after given time points.	*Plasmodium falciparum*	(Desjardins et al., 1979) (Rottmann et al., 2010)
	Incorporation of radioactive precursor into DNA, counting after given time points.	*Giardia lamblia*	(Adagu et al., 2002)
Quantitative PCR	Quantitative real-time PCR for tachyzoite DNA in infected Vero cells.	*Besnoitia besnoitii*	(Cortes et al., 2007)
	Quantitative real-time PCR for merozoite DNA in infected human ileocecal adenocarcinoma cells	*Cryptosporidium parvum*	(Arai et al., 2011)
	Quantitative real-time PCR for tachyzoite DNA in infected fibroblasts.	*Neospora caninum*	(Esposito et al., 2005)
	Quantitative real-time PCR for tachyzoite DNA in infected fibroblasts.	*Toxoplasma gondii*	(Kropf et al., 2012)

Continued

Table 7.2 Selected examples of in-vitro-screening methods for antiprotozoal compounds—cont'd

Principle	Method	Organism	Reference
Transgenic strains	Activity of glucuronidase reporter gene	*Giardia lamblia*	(Müller et al., 2009b)
	Activity of beta-galactosidase reporter gene.	*Neospora caninum*	(Howe et al., 1997; Howe and Sibley, 1997)
		Sarcocystis neurona	(Gaji et al., 2006)
		Toxoplasma gondii	(McFadden et al., 1997)
	Quantification of yellow fluorescent protein.	*Sarcocystis neurona*	(Gaji et al., 2006)
		Toxoplasma gondii	(Gubbels et al., 2003)
	Activity of luciferase reporter gene.	*Plasmodium falciparum*	(Cui et al., 2008)
	Activity of luciferase reporter gene, constitutive or schizont-specific promotor.	*Plasmodium berghei*	(Franke-Fayard et al., 2008)
Vitality	Reduction of resazurin.	*Giardia lamblia*	(Bénéré et al., 2007)
	Reduction of tetrazolium.	*Giardia lamblia*	(Wright et al., 1992)
	Quantification of ATP	*Giardia lamblia*	(Chen et al., 2011)
	Quantification of ATP	*Pneumocystis carinii*	(Chen and Cushion, 1994)
	Quantification of mRNA specific for the parasite in a coculture with host cells.	*Neospora caninum*	(Strohbusch et al., 2008)
Cell damage	Release of nucleoside hydrolase.	*Giardia lamblia*	(Kang et al., 1998)

morphological assays are easy to perform and give unambiguous information on drug effectiveness in the hands of a skilled lab worker, but they are time-consuming and rely on a "subjective" assessment. Therefore, they tend to be replaced by quantitative, "objective" methods that can, ultimately, be performed by a robot.

The simplest way to do quantify parasites is to measure a dye absorbed by the parasite by colorimetry. In the case of *G. lamblia*, trophozoites can be stained with methylene blue (Busatti and Gomes, 2007). After some washing steps, the staining is then quantified using a colorimeter. These assays are inexpensive and fast, but are limited to axenically grown parasites. Methods based on the incorporation of radioactive precursors of nucleic acids are also limited to axenically grown parasites (Adagu et al., 2002) or to co-cultures where rapidly proliferating parasites incorporate these precursors to much larger extents than their host cells (Innes et al., 1996). In the case of *Plasmodium falciparum*, intraerythrocytic stages can be stained and quantified using intercalating dyes (Banieck et al., 2007; Duffy and Avery, 2012) or radioactive precursors (Desjardins et al., 1979; Rottmann et al., 2010) since mature mammalian erythrocytes do not contain DNA.

In other cases of parasite–host cell co-cultures, parasites have to be quantified in a more specific way. A method of choice is immunofluorescence. Intrahepatocytic stages of *Plasmodium* sp. can be quantified using immunofluorescence employing an infrared fluorescence scanning system (Gego et al., 2006). An alternative is quantitative real-time PCR based on a gene specific to the parasite as implemented, e.g. for *Neospora caninum* in co-culture with human fibroblasts (Esposito et al., 2005) or for *G. lamblia* in co-culture with human Caco2 cells (Müller et al., 2006).

An elegant way to develop antiparasite drug screening assays is to use transgenic parasites expressing reporter genes under the control of a strong promotor (Rodriguez and Tarleton, 2012). The reporter genes are enzymes such as beta-galactosidase (McFadden et al., 1997), glucuronidase (Müller et al., 2009b) or luciferase (Cui et al., 2008; Franke-Fayard et al., 2008), which can easily be quantified in standard enzyme assays, or green or yellow fluorescent protein quantified using a fluorimeter (Gubbels et al., 2003). The use of transgenic parasites offers many advantages as compared to PCR-based methods. In the case of enzymes as reporter genes, the assays can be performed in situ, i.e. in 96-well plates, for instance, without prior harvest or extraction of the cells like in PCR-based methods. A second advantage is higher sensitivity. This might sound strange since PCR is often referred to as "the

most sensitive method" detecting "one copy" of a gene. This may be true under ideal conditions. One has to consider, however, that only a minor part of the DNA extracted from wells of a culture plate is analyzed by one PCR reaction. For example, if DNA from a well is eluted in 50 µl and if it contains 10 copies of the gene to be detected, the probability for 5 µl to contain 1 copy is according to the Poisson distribution 0.367 (1/e), the same as for 0 copies. To ensure a correct detection of a small number of gene copies, the PCR reactions must be upscaled by a factor of 10. On the other hand, 10 *T. gondii* or *N. caninum* tachyzoites expressing *Escherichia coli* beta-galactosidase can be detected in 96-well plates in the presence of host cells by a mere increase of the incubation time (McFadden et al., 1997; own unpublished results). A similar sensitivity is described for *T. gondii* expressing yellow fluorescent protein (Gubbels et al., 2003). The use of fluorescence has, however, the following disadvantages: i) the sensitivity cannot be extended by a mere increase of incubation time; ii) care must be taken to minimize autofluorescence by host cells, medium compounds, drug residues, etc. In some instances, cells have to be washed and overlaid with a convenient buffer. A clear advantage of strains expressing fluorescent proteins is that they allow a continuous monitoring of parasite proliferation under nondestructive conditions, even in the presence of host cells with no or little background.

In order to quantify only the viable parasites (as opposed to the overall number of parasites), vitality assays can be employed. Gene expression occurs only in viable cells, and this may qualify assays based on the incorporation of nucleic acid precursors and assays based on the use of transgenic parasites as a kind of vitality assay. The quantification of mRNA of a parasite-specific gene by quantitative RT-PCR (Strohbusch et al., 2008) has been a step in this direction since mRNA has a shorter half life than DNA. The half lives of nucleic acids and proteins are, however, much longer than the half lives of the intermediary metabolites detected in vitality assays that quantify actively metabolizing cells, only. They are based on the detection of intermediate metabolites with a rapid turnover, such as ATP using the luciferin–luciferase assay (Chen and Cushion, 1994), or NADH which is quantified with tetrazolium salts (Wright et al., 1992) or with resazurin (Bénéré et al., 2007). When employed with *G. lamblia*, the resazurin or "Alamar Blue" assay allows a rapid and reliable quantification of trophozoites grown in microtiter plates, but care must be taken to remove the reducing medium by repeated washes with prewarmed PBS since

the medium reduces resazurin and thus causing high background values (Nillius et al., 2011). In drug efficacy studies, vitality assays may lead to false-positive results due to metabolically inactive stages of the parasite that may survive the treatment and resurrect after the drug pressure has been removed.

Complementary to vitality assays are methods that monitor cell damage. As a general principle for such assays, cultures of parasites are treated with compounds, and after given time points, medium is harvested and the release of intracellular enzymes such as, e.g. nucleoside hydrolase (NH) from *G. lamblia* (Kang et al., 1998) or phosphoglucose isomerase from the helminth *Echinococcus multilocularis* (Stadelmann et al., 2010) is quantified.

2.1.2. Quantification of Drug Effectiveness

In the first round, a screen with a high concentration of test compounds allows to identify effective compounds. In the next step, the efficacy of the positively tested compounds has to be quantified in terms of inhibition constants that allow a comparison with other compounds. The constants that are most widely used are the concentration corresponding to 50% growth inhibition of the parasite (IC_{50}) and the minimum inhibitory concentration (MIC) corresponding to the minimal concentration of the compound at which growth of the parasite cannot be detected anymore (Andrews, 2001). More rarely, values corresponding to 90% inhibition are also presented (IC_{90}). These three constants are summarized in Fig. 7.1A representing an ideal sigmoid plot of a relative growth parameter p (control = 1) vs the concentration of a test compound.

From an inhibition curve of this shape, the IC_{50} value can be precisely determined by a linear logit transformation of p (x-axis) and the log transformation of the concentration (y-axis). The logit, $\ln(p/(1\ s-\ p))$, is 0 for $p = 1 - p$, or $p = 0.5$. The intersection of the line with the y-axis (ln of concentration) thus gives the IC_{50} value. By using a standard least square tool, the intersection value and standard errors can be determined. The logit-log plot of Fig. 7.1A is presented in Fig. 7.1B. The IC_{50} value is a constant that allows comparisons between different compounds in the same test system, different test systems or even with other constants such as binding constants of potential target proteins.

The MIC is more difficult to determine but is a useful indicator for subsequent in vivo or clinical trials of the antiparasitic compound. If the MIC value of a given compound in vitro is much higher than the concentration that is

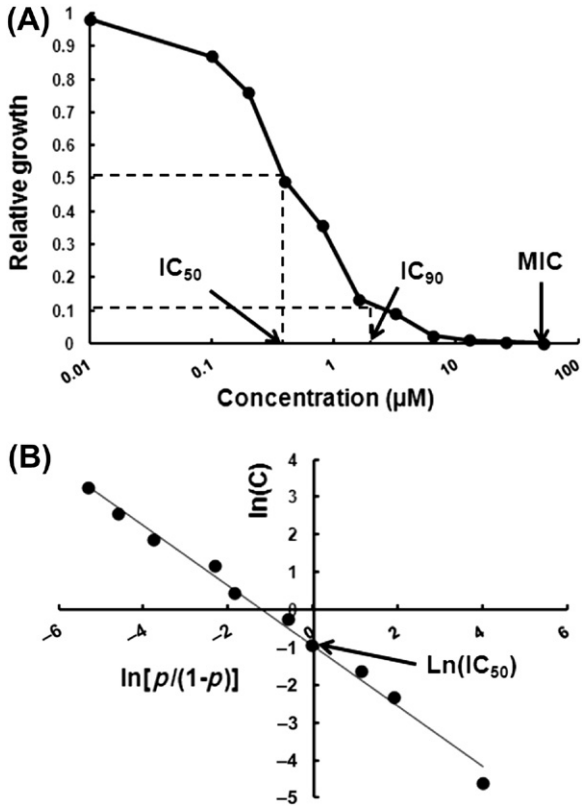

Figure 7.1 A, inhibition curve with fictive data summarizing the major constants that are used to define drug efficacy in in vitro experiments. B, logit-log plot with the same data as in A; p, relative growth parameter.

achieved in the serum of test animals or patients, this compound would not be the good choice against parasites causing systemic infections and should be eliminated from the trial, thus saving resources and test animals.

2.2. Visualization of Drug Effects on Cellular Morphology and Ultrastructure

The identification of potential mechanisms of action is often initiated by evidencing morphological and/or ultrastructural effects on the parasite at organism level (in the case of multicellular parasites), or at the cellular and subcellular levels. The major outputs are the speed and the route of the drug action. This includes data on which part or organelle of the parasite (i.e. tegument, membrane, nuclear organization, mitochondria, cytoskeleton) exhibits major alterations, and at which time point after initiation of drug treatment.

It is important to keep in mind that changes in cellular organelles and tissue structures seen at later time points during drug treatment are not necessarily linked to the action of the compound itself, but often represent simply general cell death-related alterations. Thus, corresponding assessments require experienced personnel and are, again, often "subjective." In addition, these observations will rarely provide conclusive results with respect to a drug target but can give an indication on which cellular processes are affected. Nevertheless, they may yield precious information for the discrimination of putative drug targets identified by the strategies described below (Table 7.3).

Table 7.3 Strategies for the identification of drug targets

Strategy	Suitable organisms	Input	Output
Whole organism analysis	All	Intra- or extracellular parasites treated with drug.	Spatiotemporal effects on cellular and subcellular morphology.
Drug-affinity chromatography	All	Cell-free extracts, soluble or membrane proteins.	Drug-binding proteins.
Analysis of resistant parasites	Parasites or parasite stages allowing in vitro culture and analysis of resistant strains.	Differential genome or transcriptome analysis of resistant clones.	Differentially mutated or regulated genes in drug susceptible vs resistant strains.
Genome mining	All parasites with sequenced genomes.	Class of well-known target proteins for a given drug.	Recombinant proteins expressed e.g. in *E. coli* or yeast for drug-binding studies.
Reverse genetics	All parasites that allow overexpression or silencing of genes.	Results from drug-binding studies with recombinant proteins and from analysis of resistant strains.	Overexpression of drug receptors or activators enhances susceptibility; overexpression of drug metabolizers enhances resistance (silencing: vice-versa).

2.3. Identification by Drug–Target Affinity

From the perspective of an investigator, the ideal situation for elucidating the mode of action of antiparasitic drugs consists in binding of a given compound to a target molecule (DNA, RNA or protein), and this interaction then results in the inhibition of essential cellular functions. In the case of DNA, intercalating agents are identified via their ability to slow down DNA migration in conventional gels upon electrophoresis, or via changes in their fluorescence properties (e.g. ethidium bromide is an intercalating agent). In addition, they interfere in DNA replication and RNA synthesis and/or processing and thereby inhibit protein biosynthesis. Since DNA is ubiquitous, intercalating agents are not specific and, therefore, prone to side effects on part of the host cells. Therefore, the relevance of a DNA-binding agent as an antiparasitic drug resides in other—more species specific—properties. These can include the preference for circular DNA (like the genome of mitochondria in trypanosomatids and the apicoplast in apicomplexan parasites), differential uptake or metabolism, or the presence of other targets besides DNA. These targets are identified by application of other methods.

Soluble protein targets may be identified using affinity chromatography. In such cases, the drug molecule is covalently linked to a matrix, proteins from crude parasite extracts or defined fractions are allowed to bind, and bound proteins are eluted by competition after extensive washes using unbound drug, and/or by unspecific conditions such as high salt concentrations or low pH. In order to minimize artifacts, pull-downs are performed in parallel on a mock-coupled matrix or, better, on a matrix coupled to an irrelevant compound of similar structure (Knockaert et al., 2000). The eluted proteins are conveniently identified by mass spectroscopy. The relevant proteins are then cloned, overexpressed in a suitable bacterial or eukaryotic system and purified. These expressed proteins are employed to perform binding studies or activity assays (in the case of proteins with enzymatic functions) in the presence of the drug and analogous compounds.

The advantage of this approach is that it is "hypothesis-free", i.e. we are not working with a predefined class of potential target proteins. The method can be extended to membrane proteins or even cytoskeletal proteins by adapting the extraction conditions. The disadvantage of such an approach is that irrelevant proteins may be identified, which bind to the drug matrix by mere structural coincidence but without being affected in their function. Moreover, detoxification enzymes or transporters, which preferentially bind

to xenobiotics, may be identified besides the "true" target. In some cases, susceptibility to a drug is triggered by the ability to convert an ineffective prodrug to the effective drug. In these instances, the searches for drug targets have to be performed with the effective molecules rather than with the prodrug in order to exclude that the transforming enzyme is identified rather than the target itself.

2.4. Identification of Drug Targets by Analysis of Resistant Parasites

A typical study of this type is performed by (i) creating resistant mutants of a drug-sensitive parasite, either by continuous breeding in the presence of increasing amounts of the drug or by mutagenesis followed by selection of resistant populations or clones in the presence of lethal concentrations of the drug; (ii) comparison of the resistant clones to the corresponding drug-sensitive wildtype on the genomic, transcriptomic and/or proteomic levels, and selection of differentially expressed genes/proteins; (iii) the functional analysis of differentially expressed genes by overexpression and/or silencing and after heterologous expression in a suitable system. Since point mutations causing the presence or absence of a single amino acid or nucleotide (in the case of rRNA as a target) discriminate between resistance or susceptibility, whole genome sequencing of resistant clones is necessary.

The advantage of this approach is that it is—again—hypothesis-free. The disadvantage is that it may not lead to the direct target, but to proteins involved in transport or metabolism of the (pro-)drug. An example for this is given by spiroindolone resistance in *P. falciparum* due to a mutation in a cation transporter (Rottmann et al., 2010). Moreover, due to the flexibility of gene expression in protozoa (e.g. *G. lamblia*), it is possible that the true target is hidden by resistance formation in resistant clones due to compensatory changes in gene expression (Müller et al., 2008a).

2.5. Identification of Drug Targets by Genome Mining

For many drugs in the market, the relevant target molecules are known. Due to amazing genome sequencing efforts in the years 1990–2010, whole genome sequences of parasites are available in interactive databases on the World Wide Web (e.g. www.genedb.org). These genomes can be searched for the presence or absence of potential target molecules. Following overexpression of the targets, drug-binding studies can be performed in order to obtain information on the potential activity of a given compound. Drug derivatives with a better selectivity for parasite vs host enzymes can be

designed, and assessed in similar studies, at least in theory. This approach is not "hypothesis-free" in the sense that the class of target molecules is known. The great advantage is that—once the genome information is known and accessible—this approach is very straightforward. By employing modern high-yield expression of recombinant proteins, high-throughput screening systems can be developed and compound libraries can be analyzed on several isoforms of target proteins. If the structure of the target protein is resolved, quantitative structure activity relationship (QSAR) studies can be performed predicting the interaction of potential effectors with the binding site. Since these studies are based on isolated, recombinant target proteins, a careful validation of screened compounds by in vitro and in vivo methods ("physiological screening") is paramount.

2.6. Validation of Drug Targets

In a next step, the targets identified by any of the strategies mentioned above have to be validated (Egner et al., 2005; Wang, 1997). If the drug target is a protein, this validation includes the expression of the protein in a suitable organism, followed by binding and/or inhibition studies in vitro and structural analysis of protein–ligand complexes. A bona fide drug target should have binding affinities to the drug in the same order of magnitude or lower as the inhibition constant of the drug on the parasite. If the parasites allow overexpression or silencing of genes, the drug target protein should further be validated by knockdown and overexpression studies of the corresponding gene. In particular, this approach allows distinguishing between target proteins that are directly involved in the mode of action of the drug and proteins that are only indirectly linked to the mode of action, e.g. drug metabolizing enzymes. In the first case, overexpression would enhance drug susceptibility, whereas in the latter case, overexpression would enhance drug resistance. Selected examples of studies on specific drug targets are given in the following chapters. Most studies combine the strategies mentioned above.

3. KINDS OF DRUG TARGETS

3.1. Drug Targets in the Gene-Expression Machinery

3.1.1. DNA Intercalating Agents

Among the first antiparasitic drugs are compounds that directly intercalate into DNA. The acridine derivative quinacrine, an early antimalarial and the first antigiardial drug, interacts with DNA, and thereby inhibits replication

and RNA and protein biosynthesis (Ciak and Hahn, 1967). Like other acridine derivatives, quinacrine is an intercalating agent binding to DNA with a preference for (A+T)-rich regions and thereby blocking DNA replication and RNA biosynthesis. One of the most commonly known intercalating agents, ethidium bromide, has been used since the 1950s as antitrypanosomal drug in African cattle (Roy Chowdhury et al., 2010).

The binding affinities of pentamidine derivatives (Bell et al., 1991) and bis-benzimidazoles (Bell et al., 1993) to calf thymus DNA are strongly correlated to their efficacies against *G. lamblia*. Pentamidine-derivatives such as diamidines and arylimidamides are effective not only against *G. lamblia* but also against a variety of other parasites (Torres-Gómez et al., 2008) including trypanosomatids (Shapiro and Englund, 1990; Soeiro et al., 2008, 2005) and apicomplexa, namely *N. caninum* in vitro (Leepin et al., 2008; Schorer et al., 2012) and in vivo (Debache et al., 2011), and *T. gondii* in vitro (Kropf et al., 2012). Since DNA as a target is ubiquitous, the relative specificity of DNA-intercalating agents as antiparasitic drugs must reside in other properties such as differential uptake/metabolism in parasite vs host cells. Analysis of a *G. lamblia* strain resistant against quinacrine has shown differential uptake to be a key factor of resistance formation against this intercalating agent (Upcroft et al., 1996). Moreover, the presence of circular DNA in organelles essential for the parasite such as the kinetoplast in trypanosomatids (Shapiro and Englund, 1990) or the apicoplast in apicomplexa (Dahl and Rosenthal, 2008; Fichera and Roos, 1997) may render those organisms more susceptible to these drugs than the host cells.

3.1.2. DNA Modifying Enzymes

Some intercalating agents interact not only with DNA but also with DNA modifying enzymes. This may enhance their specificity. DNA modifying enzymes may thus constitute more selective targets for antimicrobial agents. For instance, pentamidines and a variety of other DNA binding compounds, such as etoposide, induce the cleavage of trypanosome DNA minicircles in a pattern that resembles the action of topoisomerase II inhibitors (Shapiro and Englund, 1990). Fluoroquinolones, such as ciprofloxacine, inhibit DNA-gyrases and topoisomerases from prokaryotes (Kidwai et al., 1998) and are highly effective against *G. lamblia* as shown in a descriptive study (Sousa and Poiares-da-Silva, 2001), which, however, does not clarify which type of enzyme is inhibited. A phylogenetic analysis of type II topoisomerases reveals that the gene coding for the *G. lamblia* topoisomerase 2 (GlTop2) is eukaryote-like (He et al., 2005) and thus not closely related to

prokaryote topoisomerases. Moreover, fluoroquinolones are effective against some apicomplexans including *T. gondii* most probably due to interference with the prokaryote type of translation that takes place in the apicoplast (Fichera and Roos, 1997). Compounds interfering with DNA-modifying enzymes can be screened using a single molecule sensor as demonstrated for topoisomerase I (Koster et al., 2007). This method is investigated in more detail in a recent EU project developing a nanotechnology-based device for the study of drug–target interactions at the limit of sensitivity (Firman et al., 2012).

3.1.3. Transcriptional Activation or Silencing of Gene Expression as a Target

Regulation of gene expression via transcriptional activation and silencing via covalent modifications of histones represents a key event in eukaryotic development (Berger, 2002). These modifications include acetylation or deacetylation of lysine residues rendering the DNA in the vicinity of the residues more or less accessible for transcription. Histone methylation and demethylation exhibit the opposite effects and are part of the complex processes leading to chromatin remodeling (Ho and Crabtree, 2010). Enzymes involved in these steps are of increasing interest as targets for antiparasitic and other drugs (Unoki et al., 2009). Apicidin, a cyclic tetrapeptide from fungal origin, is one of the first antiprotozoal compounds characterized as a histone deacetylase inhibitor (Darkin-Rattray et al., 1996). Apicidin is highly effective (with IC_{50}s in the nanomolar range) against a panel of apicomplexans including *P. falciparum* and *T. gondii*, similar to the structurally similar HC-toxin. HC-toxin, as well as the related peptides trapoxin A and trichostatin, inhibits the proliferation of neoplastic cells and are irreversible inhibitors of mammalian histone deacetylase. Moreover, apicidin inhibits recombinant *Plasmodium* histone deacetylase and affects histone acetylation in *P. falciparum*-parasitized erythrocytes (Darkin-Rattray et al., 1996). More recently, derivatives of apicidin with even enhanced antiprotozoal properties have been synthesized and characterized with respect to binding and inhibition properties on histone deacetylases from *Eimeria* and host cells (Singh et al., 2002).

Since inhibition of host cell histone deacetylases occurs in the same range as for parasite enzymes, it remains unclear how inhibition of histone deacetylase interferes with parasite survival. One could imagine the following hypotheses in order to explain the observed effects: i) histone hyperacetylation in the parasite and the host cell leads to apoptosis of infected

host cells, whereas noninfected host cells remain nonapoptotic; ii) histone hyperacetylation itself is lethal for the parasite due to a generalized deregulation of gene expression; iii) histone deacetylase is not the only target, another—parasite-specific—enzyme system is inhibited by the same class of compounds. More recent investigations using a member of another class of cyclic tetrapeptides derived from *Acremonium* species provide novel and important insights. One of these tetrapeptides induces hyperacetylation and inhibits proliferation of *T. gondii*, *N. caninum* and *P. falciparum* with an IC_{50} of approximately 10 nM, and inhibits human foreskin fibroblasts and a resistant *T. gondii* strain at concentrations one magnitude higher. The histone deacetylase HDAC3 appears as a probable target since the recombinant enzyme is inhibited by the tetrapeptide (but with a Ki orders of magnitude higher than the in vivo IC_{50}), and since resistant mutants show distinct point mutations at a single locus in a conserved region of this gene (Bougdour et al., 2009).

Histone acetylation can be influenced also by inhibition of histone acetylation via histone acetyltransferase. A quinoline derivative inhibiting the histone acetyltransferase GCN5 from yeast does not inhibit the homologous enzyme from *T. gondii* nor does it interfere with histone acetylation of this parasite but inhibits proliferation with a moderate IC_{50} of around 100 μM. Clearly, another target must be present in this case (Smith et al., 2007).

3.1.4. Translation

Aminoglycosides such as tetracycline bind to the small subunit of prokaryote-type ribosomes, more exactly to distinct features of the 16S-rRNA secondary structure, and thereby inhibit translation. A detailed structural analysis is given by Brodersen et al. (2000). This explains why specific mutations in this region confer resistance to various aminoglycosides in various prokaryotes. *Giardia lamblia* has a 16S-rRNA structure similar to prokaryotes with a primary sequence suggesting that only paromomycin and hygromycin are effective, and other well-known aminoglycosides such as kanamycin are not. This pattern correlates well with observed susceptibilities or resistance to a panel of aminoglycosides (Edlind, 1989).

Due to its prokaryote origin, the apicomplexan plastid is a suitable target for antibiotics interfering with protein biosynthesis (Fichera and Roos, 1997; Fleige and Soldati-Favre, 2008). The aminoglycoside paromomycin is a well-established drug that is effective against intestinal infections and also moderately acts against *Cryptosporidium parvum*. However, *Cryptosporidium*

lacks an apicoplast and is regarded as a distantly related lineage that is, in fact, not coccidian, but occupies many of the same ecological niches as the other apicomplexans (Barta and Thompson, 2006). The large subunit of prokaryote ribosomes is also targeted by various antibiotics. One of these is the macrolide spiromycin, which is used in chemotherapy against *T. gondii* (Greif et al., 2001), another is the lincosamide clindamycin effective against *T. gondii* most likely due to interference with protein biosynthesis of the apicoplast (Fichera and Roos, 1997).

3.2. Cytoskeletal Proteins as Drug Targets

Integrity of the cytoskeleton is essential for maintenance and infectivity of protozoan and helminth parasites. Therefore, its components, especially tubulin, may constitute suitable targets for antiprotozoal drugs (Werbovetz, 2002). Probably, the most seriously investigated drug acting against parasite cytoskeletal components is albendazole. Molecular genetic analysis of resistant vs susceptible organisms has revealed that sensitivity to albendazole (or other benzimidazoles) in evolutionary distant organisms such as fungi, nematodes (Driscoll et al., 1989; Kwa et al., 1995), platyhelminthes (Hemphill and Müller, 2009), and various protozoa including *G. lamblia* (Katiyar et al., 1994; Kirk-Mason et al., 1988) correlates with the presence of specific alleles of β-tubulin genes. The substitution of Phe to Tyr in position 200 is sufficient for switching from benzimidazole sensitivity to resistance as shown using *Caenorhabditis elegans* as a model system (Driscoll et al., 1989; Kwa et al., 1995). Molecular modeling of the putative albendazole-binding site in albendazole-resistant *Acanthamoeba* β-tubulin indicates that four of 13 residues are different from tubulins of sensitive organisms. Resistance is conferred when, besides Phe200, Phe167 is replaced (Henriquez et al., 2008). The mode of action of the benzimidazole triclabendazole, one of the most important flukicides, is not fully understood but most likely related to the one described for albendazole (Brennan et al., 2007).

Albendazole and related benzimidazoles interact with recombinant β-tubulin monomers of sensitive protozoa including *G. lamblia* and *C. parvum* and inhibit tubulin polymerization. When added to assembled microtubules, depolymerization is, however, not induced (MacDonald et al., 2004). These molecular data suggest that albendazole has a low toxicity for mammalian host cells since they have "resistant" β-tubulin alleles. It is, however, well documented that albendazole affects growth of embryonic rodent cells (Whittaker and Faustman, 1991) and may therefore be teratogenic.

Another class of compounds interacting with the cytoskeleton is taxanes including taxol, the first natural product shown to stabilize microtubules. This unique mechanism of action is in contrast to benzimidazoles and other microtubule poisons, such as colchicine, which inhibit tubulin polymerization, and thus destabilize microtubules. In cancer cells, taxanes block cell cycle progression and sometimes trigger apoptosis (Abal et al., 2003). Taxol is also active against *Leishmania* promastigotes (Moulay et al., 1996) affecting the assembly of purified tubulin and interfering with mitosis (Havens et al., 2000). Issuing from these findings, novel antileishmanial compounds interacting with tubulin have been identified (Morgan and Werbovetz, 2008). Similar to the benzimidazoles, the major problem is to find taxanes with a high potential against *Leishmania* and a low teratogenic potential for patients.

3.3. Drug Targets in Intracellular Signaling

3.3.1. cAMP-Dependent Pathways

Intracellular signaling pathways are a source for drug target search not only in antiparasitic drug development but also in other fields including inflammation, neurodegeneration and cancer (Cunningham et al., 2008; Lugnier, 2006; Savai et al., 2010). In some lymphomas, inhibition of cAMP-dependent protein kinases or phosphodiesterases (PDEs) interferes in cAMP-related signaling pathways (Lerner et al., 2000). The classical approach is that the putative target enzymes are cloned from drug-susceptible parasites (genome mining), and inhibition constants are determined in the presence of the drug and derivatives (Liotta and Siekierka, 2010). The underlying idea is that like in cancer cells, these drugs interfere with the cAMP-dependent regulation of the cell cycle of the parasites, thereby inhibiting proliferation and/or differentiation (Hammarton et al., 2003).

Plasmodium contains a panel of cAMP-dependent and other protein kinases (Leroy and Doerig, 2008) which may constitute suitable targets for antimalarial drug development (Jirage et al., 2010). Obviously, similar studies have to be performed with enzymes from the (hopefully) resistant host in order to forecast potential side effects. In a recent study, the cAMP-dependent protein kinase PKA from *P. falciparum* has gained importance as a target since it has important differences to PKAs from mammalian hosts (Haste et al., 2012). In a study concerning the potential effects of various antimalarials on host cells, inhibition of cAMP-dependent protein kinase from rat liver and bovine heart and of calmodulin-dependent myosin light chain kinase has been studied in the presence of halofantrine and related phenantrenes. In this study, also mefloquine, allegedly acting on a

completely different target (see section 4.2.) inhibits one of those signaling proteins, namely calmodulin-dependent myosin light chain kinase with high affinity (Wang et al., 1994).

Moreover, adenylyl cyclases, the enzymes that synthesize cAMP, may be valuable targets. In *P. falciparum*, one of the two cyclases, PfACβ, plays an important role during the erythrocytic stage of the life cycle. PfACβ is susceptible to a number of small molecule inhibitors, one of them being selective for PfACβ relative to its human ortholog, soluble adenylyl cyclase (Salazar et al., 2012).

PDEs have more recently been identified as novel drug targets that exhibit a great potential as antiparasitic agents. All protozoan parasites for which genome data are available code for at least one class1 PDE. The amino acid sequences of these enzymes are reasonably well conserved among the parasites, as well as between them and the human PDE families. Considering the current wealth of inhibitors for and information about the human PDEs, the parasite PDEs are being exploited as drug targets, with medicinal chemistry developing corresponding inhibitors, which specifically target the parasite PDEs but not the human PDEs. This approach has been followed up in more depth for two related parasites: *Trypanosoma brucei* (Oberholzer et al., 2007), the causative agent of African human sleeping sickness, and *Leishmania major*, the causative agent of cutaneous leishmaniasis (Wang et al., 2007). Both organisms, as well as all their other relatives for which genome information is available, namely *Trypanosoma cruzi* (Chagas disease), *Leishmania infantum* (visceral leishmaniasis), *Leishmania braziliensis* (mucocutaneous leishmaniasis) and *Leishmania tarentolae* (a model organisms derived from lizards) code for the same set of four PDEs (Alonso et al., 2007; Kunz et al., 2005). A typical approach for studies on PDEs is to express the gene of interest in a PDE-deficient yeast strain and to characterize it by complementation of vital functions of the deficient yeast strain and by functional assays after purification of the recombinant protein as shown for three *Leishmania* PDEs (Johner et al., 2006) and for a *Trypanosoma* PDE (Alonso et al., 2007). *Leishmania* PDE1 has been crystallized and structural data for the design of novel inhibitors are available (Wang et al., 2007). Thus, PDEs from *Leishmania* and *Trypanosoma* spp. are candidates for a target-based development of drugs against these parasites (Seebeck et al., 2011).

3.3.2. Isoflavones and Receptor Kinases

Another group of compounds with antiparasite effects allegedly acting on cell signaling are isoflavones. Targets of most isoflavones are unknown,

except genistein, which is well characterized as a tyrosine kinase inhibitor (EGF receptor kinase inhibitor) in mammalian cells. This effect depends on the presence of hydroxyl groups in position 5, 7 and 4′ of the isoflavone ring, the closely related daidzein with hydroxyl groups in positions 7 and 4′ only being ineffective as tyrosine kinase inhibitor (Akiyama et al., 1987). In *G. lamblia*, isoflavones without hydroxyl groups in these positions inhibit growth (Sterk et al., 2007). Antigiardial effects of isoflavones are thus not due to similar effects as in mammalian cells.

Concerning anti-apicomplexan activities, a panel of 52 genistein derivatives with a receptor kinase as alleged target has been described with good in vitro activities against *Sarcocystis neurona*, *N. caninum* and *C. parvum* two of them being effective also against *C. parvum* in immunosuppressed gerbils (Gargala et al., 2010). The authors, however, do not address the question whether these compounds really target receptor kinases since the panel of compounds did not contain derivatives differing only in the presence of an unsubstituted hydroxyl group in position 5. Moreover, simple controls with the mother compounds genistein and daidzein are lacking (Gargala et al., 2010). Besides receptor kinases, genistein and related isoflavones with hydroxyl groups in positions 7 and 4′ bind to estrogen receptors what may lead to potential side effects (Morito et al., 2002). For the treatment of chronic parasitoses, isoflavones with a modified estrogen receptor binding site would therefore be preferred, as exemplified by an in vitro study on *E. multilocularis* and *Echinococcus granulosus*, two helminths causing chronic diseases (Naguleswaran et al., 2006).

3.3.3. Calcium-Dependent Protein Kinases in Apicomplexan Parasites

A shining example for a target-based development of antiparasitic drugs interfering with intracellular signaling is given in the case of calcium-dependent protein kinases containing calmodulin-like domains (calmodulin-like domain protein kinase; CDPK) in apicomplexan parasites. Apicomplexans contain CDPKs of a type commonly found in plants (Billker et al., 2009). *Toxoplasma* has more than 20 CDPK, *Plasmodium* and *Cryptosporidium* have fewer than half this number. Several of these CDPKs have been shown to play vital roles in protein secretion, invasion, and differentiation (Nagamune et al., 2008). These kinases have two features required for a successful target-based drug development, namely i) they are essential for the development of the parasite in the host and ii) the differences between them and their mammalian homologs are big enough to create inhibitors with a suitable therapeutic window (Jirage et al., 2010).

Bumped kinase inhibitors—ineffective against mammalian kinases—inhibit *T. gondii* calcium-dependent kinase I (TgCDPKI) at low nanomolecular levels and interfere with the infection of cells at early stages (Ojo et al., 2010). Based on these findings, a panel of pyrazolopyrimidine compounds acting as ATP competitive inhibitors has been developed and successfully tested against *T. gondii* (Johnson et al., 2012) and *C. parvum* (Larson et al., 2012). In *Plasmodium* sp., this class of inhibitors does not affect intra- and extraerythrocytic stages in humans, but inhibits the sexual stages, namely microgametocyte exflagellation, oocyst formation and sporozoite production, necessary for transmission to mammals, in mosquitoes (Ojo et al., 2012). In combination with drugs active against blood and/or liver stages of *Plasmodium* in humans, these inhibitors may provide an important tool for malaria control and eradication by stopping genetic recombination and the transmission from infected to healthy persons.

What is the role of these kinases? In *T. gondii*, KT5926, a specific inhibitor of CDPKs, blocks the motility of tachyzoites and their attachment to host cells by inhibiting only a single KT5926-sensitive protein kinase activity, TgCDPK1, a kinase with three major substrates (Kieschnick et al., 2001). Based on these observations, a central role is suggested for TgCDPK1 in regulating *Toxoplasma* motility and host cell invasion with the myosin XIV motor complex as a probable target (Gilk et al., 2009). In *Plasmodium* sp., CDPK1 phosphorylates two components of the motor complex in schizonts, thus in asexual stages (Green et al., 2008). A better candidate for the inhibitors mentioned above is CDPK4, or a related kinase. Genetic studies show that this kinase is a molecular switch essential for the sexual reproduction and mosquito transmission of *Plasmodium berghei* (Billker et al., 2004). These examples suggest that inhibition of CDPK1 in apicomplexan parasites interferes with host cell invasion. Conversely, intracellular stages would not be affected. Therefore, additional compounds would be needed in order to cure these stages.

3.4. Membrane Integrity as a Drug Target

In the previous sections, we have provided information on the identification of proteins or nucleic acids as drug targets. Some very powerful antimicrobial compounds do, however, not target these macromolecules, but destroy the membrane integrity, and thereby cause cell death. These compounds are, in general, weak acids that, in the protonated state, freely diffuse through membranes. As a consequence, within minutes after addition of such a compound to a cell, the transmembrane proton or ion gradients are destroyed and essential cellular functions such as ATP biosynthesis are

blocked. Historically, these substances are called "uncouplers" since they facilitate the passage of protons through the mitochondrial membrane without ATP biosynthesis. Free fatty acids are well-understood examples of chemical uncouplers with interesting functions in mammalian mitochondria (Di Paola and Lorusso, 2006). Depending on their chain length and degree of saturation, fatty acids are also effective against a variety of bacteria and against *G. lamblia* (Rayan et al., 2005). Dodecanoic ("lauric") acid accumulates within minutes in *G. lamblia* trophozoites followed by the rupture of the membrane within hours (Rayan et al., 2005). Many other natural compounds with similar properties may also act on the membrane integrity by similar mechanisms (Anthony et al., 2005). Moreover, the antihelminthic salicylanilide closantel and related compounds are proton ionophores (Martin, 1997).

The membrane system is a direct target for drugs that are incorporated into the membrane bilayer because they share similarities with endogenous membrane components. A well-studied example is the polyene macrolide amphotericin B. Amphotericin B is an antifungal compound binding to ergosterol in the cell membrane, and thus causes membrane depolarization and cell damage by oxidative stress (Blum et al., 2008). Another drug directly affecting membrane integrity is miltefosine, a membrane active alkyl phospholipid, originally described as an anticancer drug and used since the early 1990s against leishmaniasis alone or in combination with amphotericin B (Moore and Lockwood, 2010).

A much more specific mode of action is observed with drugs acting on plasma membrane ion channels. A well-studied example is the antihelminthic praziquantel. Praziquantel causes a rapid uptake of calcium ions, probably by affecting voltage-gated channels (Jeziorski and Greenberg, 2006). As a consequence, the neuromuscular transmission of the helminth is affected causing tegument disintegration (in the case of *Schistosoma*) and rapid death. Another example for drugs affecting membrane proteins are quinolones like atoquanone, a ubiquinone analogon targeting the mitochondrial cytochrome bc1 complex in *Plasmodium* thereby inhibiting the respiratory chain. Resistance formation occurs, however, rapidly (Winter et al., 2008).

3.5. Drug Targets in Intermediary Metabolism

3.5.1. Glucose Metabolism

Although ubiquitous, the metabolism of glucose via glycolysis has gained some importance for the development of antiparasitic drugs. In *Plasmodium* sp., the hexose transporter (HT) is a promising candidate for the

development of novel drugs (Patel et al., 2008; Slavic et al., 2011). Uptake of hexoses by the hexose transporter from *P. falciparum* (PfHT) expressed in *Xenopus* oocytes is inhibited by 3-O-alkyl-D-glucose analogs, whereas hexose transport by the major mammalian glucose and fructose transporters remains unaffected. Inhibition of PfHT in vitro is correlated with inhibition of *P. falciparum* in culture medium containing either glucose or fructose as a carbon source, as well as with multiplication of *P. berghei* in a mouse model (Joet et al., 2003). After molecular characterization of the hexose transporter from *Plasmodium vivax*, a novel glucose analog inhibiting isolates from patients was identified (Joët et al., 2004). Moreover, 2-O-alkyl-glucose analogs inhibit PfHT (Ionita et al., 2007) and 4-O and 6-O analogs, the hexose transporter from *Babesia bovis* (Derbyshire et al., 2008). These studies are based on the expression of recombinant HTs in *Xenopus* oocytes. Another, perhaps more appropriate system, especially for drug screening purposes, is the expression of recombinant HTs in a glucose transporter null mutant of *Leishmania mexicana* (Feistel et al., 2008).

Another suitable target in glucose metabolism is glucose-phosphate-isomerase (GPI). In *T. gondii*, genes encoding GPI and another glycolytic enzyme, enolase, are highly homologous to higher plant genes and differ from those of animals. Moreover, they are expressed in bradyzoites, a stage refractory to drug treatment. They thus might offer novel chemotherapeutic targets (Dzierszinski et al., 1999). As for the glucose transporter, monosaccharide derivatives would be suitable inhibitors. Recombinant GPI from *T. brucei* is inhibited by a series of arabinose derivatives, a pentose with a spatial structure similar to fructose, one of them, D-arabinonhydroxamic acid-5-phosphate, having an IC_{50} as low as 50 nM (Hardré et al., 2000). More recent results obtained with GPI from *Eimeria* sp. suggest that these results could be extended to other apicomplexan parasites (Loo et al., 2010).

3.5.2. Prokaryote-like Pathways in Apicomplexa

Intermediary metabolism of pathogens provides suitable target options in all cases where a pathway is essential for the pathogen and is absent, or of minor importance, for the host cell. Besides traditional knowledge of intermediary metabolism of parasites, genome mining is a new, powerful tool for drug target search in this field. Prokaryote-like metabolic pathways of apicoplasts provide some good examples for this approach (Dahl and Rosenthal, 2008; Fichera and Roos, 1997).

The apicoplast organelle exhibits a type of fatty acid biosynthesis resembling biosynthetic pathways of prokaryotes and plastids from higher

plants (type II) and is therefore a highly suitable drug target (Goodman and McFadden, 2007). Like in higher plants, key enzymes of this pathway are encoded in the nucleus, synthesized in the cytosol, and targeted to the plastid (Waller et al., 1998). Thiolactomycin, a potent inhibitor of bacterial/plastid fatty acid biosynthesis, inhibits growth of *P. falciparum* (Waller et al., 1998). Conversely, *C. parvum*, lacking the apicoplast, has a conventional type I fatty acid biosynthesis pathway and is resistant to thiolactomycin (Zhu et al., 2000).

Folic acid biosynthesis is another example of a prokaryote-like pathway in apicomplexa, and thus represents an interesting drug target. Sulfonamides are inhibitors of H2-pteroate synthase and the compounds pyrimethamine and trimethoprim inhibit dihydrofolate reductase. Alone or in combination with sulfonamides, these drugs constitute the main current therapy against *T. gondii* (Greif et al., 2001).

Toltrazuril, a triazinone derivative with a broad spectrum of efficacy against cyst-forming and non-cyst-forming apicomplexa, is used as a drug against various coccidioses in livestock. Toltrazuril inhibits dihydroorotate dehydrogenase and thereby pyrimidine biosynthesis. Moreover, there is some evidence that it affects the respiratory chain. In mice artificially infected with *N. caninum*, toltrazuril and its main metabolite ponazuril have been shown to reduce clinical signs and cerebral lesions and to interfere with the diaplacental transmission of *N. caninum* tachyzoites to newborns (Gottstein et al., 2005). Moreover, toltrazuril is effective in the treatment of congenitally *Neospora*-infected newborn mice (Strohbusch et al., 2009). The observations from this mouse model can, however, not be extended to the economically more relevant situation in cattle since toltrazuril treatment does not reduce seropositivity in congenitally infected calves (Haerdi et al., 2006).

3.5.3. Nitro Compounds Effective against Microaerophilic or Anaerobic Parasites

The nitroimidazole metronidazole is clearly one of the best-studied compounds affecting intermediary metabolism in anaerobic or microaerophilic protozoa like *Entamoeba histolytica*, *G. lamblia*, or *Trichomonas vaginalis* (Löfmark et al., 2010). Formation of resistance to metronidazole (Upcroft and Upcroft, 1993) is associated with an altered uptake of the drug (Boreham et al., 1988), and downregulation of pyruvate:flavodoxin/ferredoxin oxidoreductase (PFOR) activity, a protein lacking in higher eukaryotic cells (Horner et al., 1999). Based on these results, the general view is that

metronidazole (as a prodrug) is reduced to a nitro radical (Sisson et al., 2002) by electrons coming from PFOR (Upcroft and Upcroft, 2001). This radical causes irreversible damage on intracellular structures. More recent findings in the microaerophilic parasite *T. vaginalis* indicate, however, another mode of action. By comparing 2-D-protein electrophoresis patterns from treated and untreated cells, the authors suggest that metronidazole binds to the sulfhydryl group in the active center of various enzymes including thioredoxin reductase thereby impairing essential cellular functions, and that resistant cells overcome this blocking by re-regulating other enzymes involved in oxidoreductive processes, such as PFORs (Leitsch et al., 2009a). In this model, downregulation of PFOR would be a consequence rather than a prerequisite of resistance formation. Also in this model, an activated form of metronidazole would act on multiple targets, the number being limited to enzymes with sulfhydryl groups in their active center. Moreover, metronidazole is thought to form adducts with free cysteine since addition of free cysteine to the culture medium renders *E. histolytica* more resistant to metronidazole (Leitsch et al., 2009b). In a study on *G. lamblia*, even a positive correlation between metronidazole-resistance formation and PFOR mRNA quantity has been observed (Argüello-García et al., 2009). A more likely direct target of nitro drugs in anaerobic parasites are nitroreductases (NRs), as identified in *G. lamblia* (Müller et al., 2007b; Nillius et al., 2011). Furthermore, possible giardial metronidazole-resistance mechanisms involving complex changes in the gene expression pattern have been described, including alterations of those genes encoding variant surface proteins (VSPs), which mediate antigenic variation (Müller et al., 2007a).

3.5.4. Nitro Compounds Effective against Aerobic Parasites

Interestingly, trypanosomal mitochondria contain a bacterial-like NR activating the antitrypanosomal prodrugs benznidazole and nifurtimox, loss of a single copy of NR in *T. cruzi* being sufficient to cause significant cross-resistance to these drugs and to other nitroheterocyclic compounds (Wilkinson et al., 2008). The recombinant NR from *T. cruzi* and *T. brucei* are used in target-based screenings in order to identify novel antitrypanosomal agents (Hall et al., 2010). Trypanosomal NR catalyzes an oxygen–insensitive reaction with the interaction of enzyme, reductant, and prodrug occurring through a ping-pong mechanism. In the case of benznidazole as a prodrug, the major product is 4,5-dihydro-4,5-dihydroxyimidazole releasing the reactive dialdehyde glyoxal during his further breakdown. In the presence of guanosine, cytotoxic guanosine-glyoxal adducts are generated (Hall

and Wilkinson, 2012). Benznidazole resistance in *T. cruzi* is correlated with independently acquired mutations in the NR gene (Mejia et al., 2012), a fact that further sustains the role of NR as the major target for benznidazole and related compounds in trypanosoma.

3.5.5. Radical Formation in Aerobic Parasites

The formation of toxic radicals, but under aerobic conditions, is described as the mode of action of artemisinin and its derivatives (Asawamahasakda et al., 1994), which represent a class of well-investigated antimalarial drugs (Cui and Su, 2009). The mechanism relies on the cleavage of the endoperoxide bridge of the molecule resulting in the generation of an unstable oxygen-centered radical. This radical subsequently alkylates various enzymes of intermediary metabolism and causes membrane damage. The activation of the molecule occurs in mitochondria from *Plasmodium* and lower eukaryotes such as yeast, probably via the xanthine oxidase system, but not in mammalian mitochondria, which may explain the specificity of the drug (Wang et al., 2010). Intracellular heme seems to be the physiologically relevant mediator of artemisinine action (Zhang and Gerhard, 2009). A relevant target protein is plasmepsin II, a heme degrading enzyme of *Plasmodium* sp., as shown in a QSAR study supported by in vitro and in vivo tests of the screened compounds (Qidwai et al., 2012). Besides *Plasmodium* sp., artemisinin inhibits proliferation of *N. caninum*, an apicomplexan closely related to *T. gondii*, in Vero cells with little or no host-cell toxicity (Kim et al., 2002).

3.5.6. The Purine Salvage Pathway

Unlike their mammalian hosts, many protozoan parasites are unable to synthesize purines *de novo* and have to recover them from nucleosides present in the medium (Datta et al., 2008). One way is the cleavage of nucleosides by a less or more specific NH into purine or pyrimidine bases and (desoxy) ribose. *Giardia lamblia* NH interacts with isoflavones as found during an affinity chromatography study and thus may be a target for these compounds (Sterk et al., 2007). Yeast expressing *L. major* NH constitute a suitable system for screening inhibitors of this enzyme and thus, potential antileishmanial compounds (Miller et al., 2007). In *Plasmodium* sp., the purine salvage pathway consists of adenosine deaminase, purine nucleoside phosphorylase (PNP) and hypoxanthine–xanthine–guanine–phosphoribosyltransferase (HGXPRT). Genetic studies have confirmed the importance of *Plasmodium* PNP for viability of malaria parasites. *Plasmodium* PNPs are inhibited by immucillin, some of which having undergone human clinical trials. These

compounds thus represent a new class of compounds with potential activity against human malarias (Donaldson and Kim, 2010). Related compounds, acyclic immucillin phosphonates, inhibit HGXPRT (Hazleton et al., 2012). As a result, imucillin derivatives may inhibit the purine salvage pathway of *Plasmodium* at two different steps and thus, constitute valuable compounds against malaria.

4. CASE STUDIES

In the following sections, we will illustrate the problems concerning drug target identification with two case studies on antiprotozoal drugs with a broad host spectrum, namely the thiazolides with the mother compound nitazoxanide, and mefloquine, originally developed as an antimalarial.

4.1. Thiazolides, a Novel Drug Family

Since reports on novel antiparasitic drugs are rare, the release of a class of compounds, thiazolides, has been followed with great interest since the 1990s (Hemphill et al., 2006, 2007). Nitazoxanide, the mother compound of this class, has been approved in the USA for the treatment of persistent diarrhea due to cryptosporidiosis and giardiasis in children and adults, and there are a couple of clinical studies with unclear readouts (Hemphill et al., 2007). Upon oral uptake, nitazoxanide is rapidly deacetylated to tizoxanide and further metabolized to tizoxanide-glucuronide (Broekhuysen et al., 2000). Tizoxanide has been reported to display antimicrobial activity similar to nitazoxanide, while tizoxanide-glucuronide is largely inactive against a number of pathogens (Adagu et al., 2002). In anaerobic or microaerophilic pathogens like *G. lamblia* or *T. vaginalis*, the presence of a nitro group is a prerequisite for efficacy (Adagu et al., 2002; Müller et al., 2006), and the mode of action is claimed as being similar to other nitro compounds like metronidazole with PFOR as a potential target. By affinity chromatography using tizoxanide as a ligand, we have pulled down an NR as a tizoxanide-binding protein from *G. lamblia* extracts. Activity of the recombinant enzyme is inhibited by nitazoxanide (Müller et al., 2007b). Overexpression of the corresponding mRNA in *G. lamblia* and *E. coli* is correlated with an increased susceptibility to nitazoxanide and metronidazole (Nillius et al., 2011), lower expression levels in a *G. lamblia* clone are correlated with resistance (Müller et al., 2008a, 2007a; Nillius et al., 2011). Thus, this NR behaves as a true target of nitazoxanide and metronidazole.

Concerning apicomplexan parasites, several studies have shown that not only nitazoxanide but also some of its derivatives lacking the nitro group exhibit in vitro activity against the intracellular apicomplexan parasites *N. caninum* (Esposito et al., 2007), *C. parvum* (Gargala et al., 2010), *Besnoitia besnoiti* (Cortes et al., 2007), *Leishmania* (Zhang et al., 2010), *Plasmodium* sp. and *Trypanosoma* sp. (Navarrete-Vazquez et al., 2011). The same affinity chromatography approach as with *G. lamblia* has been performed with *N. caninum*, and a protein disulfide isomerase, a protein with affinities to a variety of hydrophobic compounds including hormones (Primm and Gilbert, 2001), has been identified as nitazoxanide-binding protein (Müller et al., 2008b). Thus, a clear target for thiazolides in apicomplexan is still unknown.

Nitazoxanide and some non-nitro-derivatives are, however, toxic to proliferating host cells. By affinity chromatography, glutathione-S-transferase P1 (GSTP) has been identified as a thiazolide-binding protein in Caco2 cells, and inhibition of GSTP activity by nitazoxanide and some non-nitro-thiazolides is well correlated with the efficacy of these drugs to induce apoptosis. Overexpression and downregulation of GSTP is correlated with an increase or decrease of sensitivity, respectively (Müller et al., 2008c). GSTP regulates cellular stress responses and apoptosis by sequestering and inactivating c-Jun N-terminal kinase (JNK). Thiazolides induce expression and activation of a critical downstream target of JNK, Bim, in a JNK-dependent manner. If Bim is downregulated, thiazolide-induced apoptosis is blocked (Sidler et al., 2012). Sublethal concentrations of thiazolides enhance the proapoptotic effect of tumor necrosis factor.

Moreover, in human foreskin fibroblasts, quinone reductase 1 has been identified as a thiazolide-binding protein (Müller and Hemphill, 2011b). These findings suggest that NTZ and related thiazolides exert their effects against intracellular protozoa indirectly by activating innate immune responses in the host cells rather than directly by affecting the pathogen itself.

4.2. Mefloquine, Another Compound with Different Targets

The quinolines mefloquine, chloroquine and related compounds are well-established antimalarials (Brocks and Mehvar, 2003; Milner et al., 2010). Their mode of action is reported to rely on the interference with hemoglobin digestion by the parasite (Foley and Tilley, 1998). Mefloquine is effective not only against *Plasmodium* sp. but also against *Schistosoma mansoni* adult worms, where it inhibits the formation of hemozoin, a heme detoxification aggregate (Oliveira et al., 2004), and against juvenile stages (Keiser

et al., 2009). Thus, for mefloquine, there must be other targets besides heme degradation. Mefloquine and other quinolines inhibit various protein kinases but at relatively high concentrations (Wang et al., 1994). Mefloquine inhibits the proliferation of *N. caninum* and *T. gondii* tachyzoites cultured in human foreskin fibroblasts, however, the therapeutic window is very small (Müller and Hemphill, 2011a). Therefore, a target in human cells should exist. Affinity chromatography employing epoxy-sepharose-bound mefloquin and extracts of HFF resulted in the pull-down of nicotinamide phosphoribosyltransferase (accession no NP_005737) as a mefloquine-binding protein (authors, unpublished data). Further studies are required to elucidate the role of this enzyme in the mode of action of mefloquine. If human nicotinamide phosphoribosyltransferase, a key enzyme for the biosynthesis of NAD, is a true target for mefloquine, this may explain the side effects of this drug, especially in the central nervous system, as shown experimentally in rats (Dow et al., 2006) and in a survey study in humans (Barrett et al., 1996). In *S. mansonii*, the same approach has yielded enolase as a mefloquine binding protein. Mefloquine inhibits enolase activity in crude extracts, but has no effect on the recombinant enzyme expressed in *E. coli* (Manneck et al., 2012).

Taken together, both case studies show that broad-spectrum antiprotozoal drugs may interact with multiple proteins or even other macromolecules including such from host cells. Therefore, care must be taken concerning the assertion of drug target functions to macromolecules identified by a single approach. Only via a multidimensional approach including the analysis of resistant strains, overexpression and downregulation of potential target proteins in suitable cellular contexts, such an assertion *bona fide* can be made.

5. CONCLUSIONS AND OUTLOOK

In this chapter, we have provided an overview on the identification of antiprotozoal drug targets. Despite an increasing effort on target-based drug discovery strategies, all antiprotozoal drugs currently available on the market, as well as novel promising drugs (Rottmann et al., 2010), have been derived from whole organism screenings. Therefore, many drug targets and modes of action described in this chapter have been discovered after the corresponding compound has been launched. Target-based drug discovery has not been successful in terms of introducing novel antiparasitic drugs. One possible explanation for this is that target-based drug development is

powerful in the case of diseases caused by mutations in single genes but has only limited impact in cases of diseases where multiple genes (i.e. potential targets) are involved as it is the case, e.g. for obesity, and mental disorders (Sams-Dodd, 2005). Target-based drug development against parasites may thus be successful where products of single genes are involved in the mechanism of action, e.g. enzymes activating prodrugs. In this case, however, rapid resistance formation may occur.

In most of the other cases of antiparasitic drug targets, we do not fully understand the physiological responsiveness of the parasite in the presence of the drug. In the case of intracellular parasites, reactions of the host cell render the situation even more complex. As a consequence, the general concept of antiparasitic drug development should be refined. Whole organism screenings are too tedious, and for target-based screenings, the base of knowledge is still too narrow, except in some very promising cases where target molecules that are essential for the parasite, but absent in the host, have been identified. Therefore, a third strategy that combines the positive aspects of both approaches should be chosen as follows.

In the first step, libraries of anti-infective or anticancer compounds are screened using in vitro systems with the parasite of interest. For instance, since some apicomplexan parasites circumvent host cell death by secreting proteins that interfere with apoptotic pathways (Dobbelaere et al., 2000; Lang et al., 2007), compounds inducing apoptosis (e.g. derived from anticancer research) may be suitable candidates for drugs against these parasites. Successfully tested compounds will then be tested in animal models. This step is similar to the starting point of whole organism screenings, except the fact that compounds with known effects are screened. If the drug target is known from other systems, the homologous protein is identified in the parasite of interest, expressed in a suitable system, purified and subjected to binding assays with effective and ineffective compounds. If there is a correlation between growth inhibition and target binding, the target can be validated by overexpression and silencing experiments.

If the target is unknown, identification will be performed by the methods discussed above. In the case of intracellular parasites, the target search should be extended to the host cells. In a second round, trained chemists may synthesize even more effective derivatives of the compounds having successfully past the first round, and a second round of test series may follow. Then, the most effective compounds are subject to in vivo trials in a suitable animal model followed by preclinical and clinical studies.

The most promising situation for antiparasitic drug development is given when the drug target is localized in an organelle that is absent from the host cell. For apicomplexan parasites, the prokaryote-like gene expression system of the apicoplast provides highly interesting targets for antibiotics binding to DNA or ribosomal RNA. The tetracycline derivative doxycycline and the lincosamide clindamycin are, for instance, effective as antimalarials. These antibiotics may constitute interesting candidates for anti-apicomplexan drug development and will deserve further attention.

There is more and more evidence that compounds acting on ubiquitous targets such as intercalating agents, histone-modifying enzymes, compounds generating free radicals, and others will provide the basis for the development of highly efficient drugs with a large therapeutic window. The reason for this specificity is unknown and provides an interesting field for further investigations. Moreover, the case studies reported above have shown that many drugs will not only act on one target, and several drug targets can be identified for a given compound, depending on the approaches used. Action on multiple targets is, perhaps, even a prerequisite for a high efficacy of antiparasitic drugs, e.g. via a simultaneous inhibition of vital parasite functions and induction of apoptosis of host cells in the vicinity of the parasite. In addition, this would also slow down resistance formation. If this is the case, the target-based drug development paradigm (Sams-Dodd, 2005) will be even more questionable in the case of antiparasitic drugs, and development based on physiological screenings as shown by Rottmann et al. (2010) will prevail.

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